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CANCER RESEARCH

VOLUME 9 JULY 1949 NUMBER 7

Infiltrating Adenomatous Lesions of the Stomach, Cecum, and Rectum of Monkeys Similar to Early Human Carcinoma and Carcinoma *in situ**

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The experimental production in animals of neoplastic gastro-intestinal lesions of types common in man has proven extremely difficult and, in the case of most lesions, rarely successful. Spontaneous gastro-intestinal diseases in animals, clinically and pathologically similar to those of man, are also quite unusual. Only recently has experimental carcinogenesis in the intestine of rodents been successful with sufficient constancy to be of aid in the study of the pathogenesis of cancer in this site (1, 2). Even so, few adenocarcinomas of the stomach (3, 4), colon (2), and rectum of animals have been produced experimentally. Therefore the repeated, facile production of carcinoma-like lesions in the stomach (5), colon, and rectum of monkeys who had ingested motor lubricating oil is of great interest. The novelty of these findings in this species is affirmed by the few spontaneous carcinomas that have been reported in monkeys (6, 7) and by the apparent resistance of monkeys to the action of carcinogens even through 10 years of continuous treatment (7).

The observations recorded here extend those previously made, reporting the occurrence in *Macaca mulatta* (rhesus) monkeys of a hyperplastic gastritis characterized by invasion of the submucosa by proliferating hyperchromatic glands (5). These lesions were similar to those produced by Bonne and Sandground (8) in Javanese monkeys by a parasitic nematode. No gastric parasites, however, were found in the lesions in the rhesus monkeys and the responsible etiological agent in these cases seemed to be an oil to which the monkeys were exposed for several months. The impression that this oil was derived from shale and contained substances similar chromatographically to known carcinogens was corrected recently by a careful chromatographic study of this oil using more refined instruments and techniques (9). The oil, a common diesel motor lubricant, used by the Chemical Corps, U.S. Army, for producing smoke screens, was found to be free of any known carcinogenic substances. The gastric lesions previously described (5) were found unexpectedly in the course of experiments investigating the dangers of inhaling mists of the oil. The etiological role of the oil could only be assumed in the absence of any knowledge of the spontaneous occurrence of this lesion. Before additional monkeys became available for study of the effect of the oil specifically on their gastro-intestinal tract, rats were fed this oil in synthetic diets. This procedure resulted in the production of a hitherto undescribed adenomatous lesion of the colon of the rats that was quite similar to that in the stomach of the monkeys in appearance and in the manner in which the hyperplastic glands invaded the submucosal tissues (9).

The observations reported here deal with (a) the spontaneous occurrence of this type of lesion in rhesus monkeys, (b) two additional methods by

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which the lesion can be produced, (c) the role of the oil and the diet in its production, and (d) the nature of the lesions.

EXPERIMENTAL PROCEDURES

Thirty-six rhesus monkeys weighing between 4 and 5 kilograms were used in these experiments. Three experiments were performed: in the first, 22 monkeys used by other investigators for various purposes were necropsied and the gastro-intestinal tracts carefully investigated for the presence of any disease. All abnormal appearing areas of the stomach, colon, and rectum were taken for microscopic study along with sections removed routinely in all cases. In the stomach the following sites were routinely studied: the pyloric antrum and cardia along the lesser curvature; the junction of the cardia and fundus and of the fundus and antrum along the greater curvature; and fundus from the posterior and anterior surfaces. Other routine sections were: cecum, ascending colon, rectum, and ano-rectal mucosal junction. All tissues were fixed in formalin, imbedded in paraffin and stained with hematoxylin and eosin.

The second experiment comprised an attempt to reproduce the lesions originally found in monkeys exposed to aerosols of oil. Since it was felt that this lesion was the result of the oil being ingested from their bodies during the constant grooming that is done by this species, 10 ml. of oil were sprayed onto the chest and abdomen of 5 monkeys daily until the animals became moribund, a period ranging from 100 to 213 days. Five other monkeys served as untreated controls and were killed simultaneously with the moribund treated monkeys. Microscopic sections were taken and prepared as in the previous experiment.

In the third experiment 4 monkeys averaging 3.3 kg. were used. Instead of the usual diet of dog chow and cabbage, these monkeys received synthetic diets.[1] Two monkeys ate the normal diet and the other two ate a diet containing no protein in order to determine whether protein inanition which accompanies intoxication with the oil could alone produce the hyperplastic infiltrative gastro-intestinal lesions. After 6 months on these diets, the former animals had gained one kilogram each and the other two had lost a similar amount. One of the latter was moribund at that time and was killed and necropsied. The other monkey on the protein-free diet was then placed on the normal diet and the monkeys on that diet changed to the protein-free diet to which 0.85 ml. of oil were added per 100 grams of diet. One of the monkeys ingesting the oil diet died in 48 days and the other died in 78 days after losing 0.7 kg. and 1.2 kg. respectively. The remaining monkey on the normal diet gained 1.6 kg. in 78 days and was then killed and necropsied. Dietary consumption could not be followed in this experiment because the monkeys scattered the crumbly diets widely.

[1] Diet composition. *Normal diet* per 100 grams of solid: Casein, 18 gm.; gelatin, 1 gm.; sugar, 5 gm.; corn starch, 64 gm.; salt mixture, 4 gm.; ruffex, 2 gm.; liver concentrate, 1 gm.; corn oil, 3 gm.; cod-liver oil, 2 gm. This was mixed with 80 ml. of water along with: thiamine, 20 mg.; niacin, 100 mg.; riboflavin, 20 mg.; pyridoxine, 20 mg.; calcium pantothenate, 60 mg.; and choline chloride, 1 gm. *Protein-free diet* was the same except that the 18 gm. of casein was replaced by 18 gm. of corn starch. Both diets were partially dried at 60° C. in a moving current of air for 3 to 4 hours and then cut into 2 inch squares one-half inch thick. Each monkey received 200 gm. of these diets daily along with 25 gm. of fresh cabbage.

RESULTS

Incidence of gastro-intestinal disease in monkeys not ingesting oil.—Of the 22 monkeys examined, all had normal intestines and 13 had entirely normal stomachs. Seven had varying degrees of atrophic gastritis which in 5 cases was focal and minimal. In 2 cases only occasional parietal cells could be found, the mucosa was thin, and the glands were separated by a lamina propria rich in lymphocytes and containing an increased number of follicles. One apparently normal monkey that in another experiment had lived for over one year in a mist of triethylene glycol had a nodular abnormality of the fundal mucosa. Microscopically this portion of the stomach contained in a fibrotic submucosa large cystic glandular spaces with papillary projections (Fig. 1). The overlying mucosa was relatively normal. A stain for lipase indicated that these structures were composed of normal mucous neck and zymogen-containing cells. These glands were in continuity with the surface crypts. Another monkey that was ill for 3 months following the implantation of an iron ring beneath the scalp in another experiment died with a constricting lesion of the gastric antrum. Microscopically there were cystic glands in the basilar portions of an extremely hyperplastic but simplified mucosa. These cystic glands penetrated the muscularis mucosae in numerous places and formed multiple large cysts in the submucosa (Fig. 2). The external muscular layers were not invaded. The borders of the lesion, which grossly measured 4 cm. in length and encircled the stomach, were composed of hyperplastic epithelial cells that thickened the normal rugae. In these areas occasional examples of early penetration of the submucosa were found (Figs. 3 and 4). Only one monkey had demonstrable intestinal parasites and the gastro-intestinal tract of this animal was normal.

The effect of ingesting lubricating oil placed on the skin.—The 5 control monkeys in this experiment had entirely normal gastro-intestinal tracts. The 5 animals squirted daily with oil had various mucosal abnormalities. None was entirely normal. Monkey 1, dead in 100 days, had an atrophic gastritis (Fig. 5) and the anorectal junction had thickened epithelial folds composed of glands in which the cells were crowded and relatively hyperchromatic. No invasion of the submucosal structures was found. Monkey 2, dead after 120 days, had a hyperplastic gastric mucosa composed

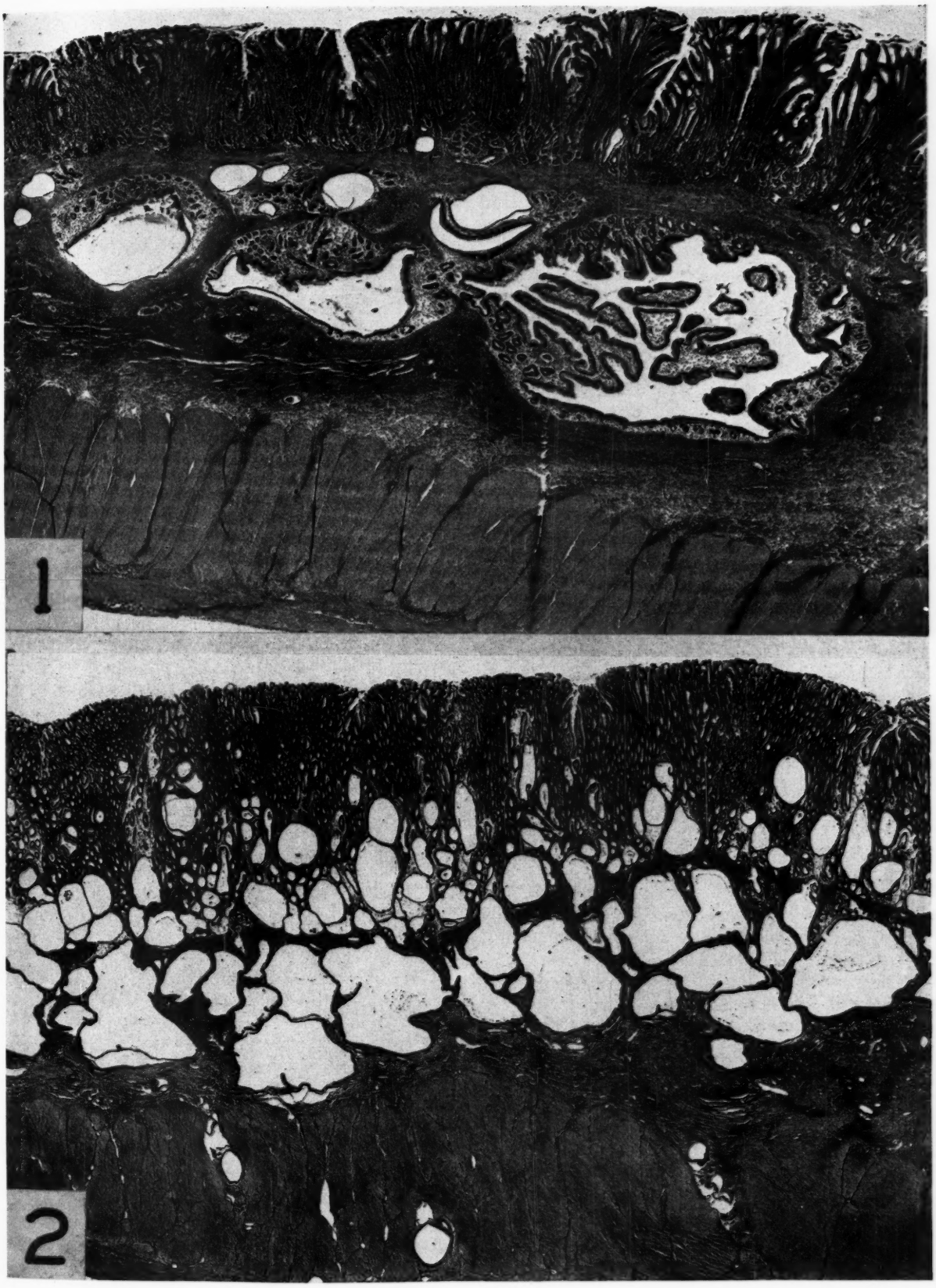


FIG. 1.—Photomicrograph of a portion of the gastric lesion in a monkey that lived about 1 year in an atmosphere containing triethylene glycol, showing large submucosal nests of cystic glands with papillomatous processes. Gomori's hematoxylin, eosin lipase stain. Mag. ×18.

FIG. 2.—Photomicrograph of the central portion of the obstructing lesion in the pyloric antrum of a monkey ill without apparent cause for 3 months, showing hyperplastic epithelium with cystic glands penetrating and filling the submucosa. Hematoxylin and eosin stain. Mag. ×15.

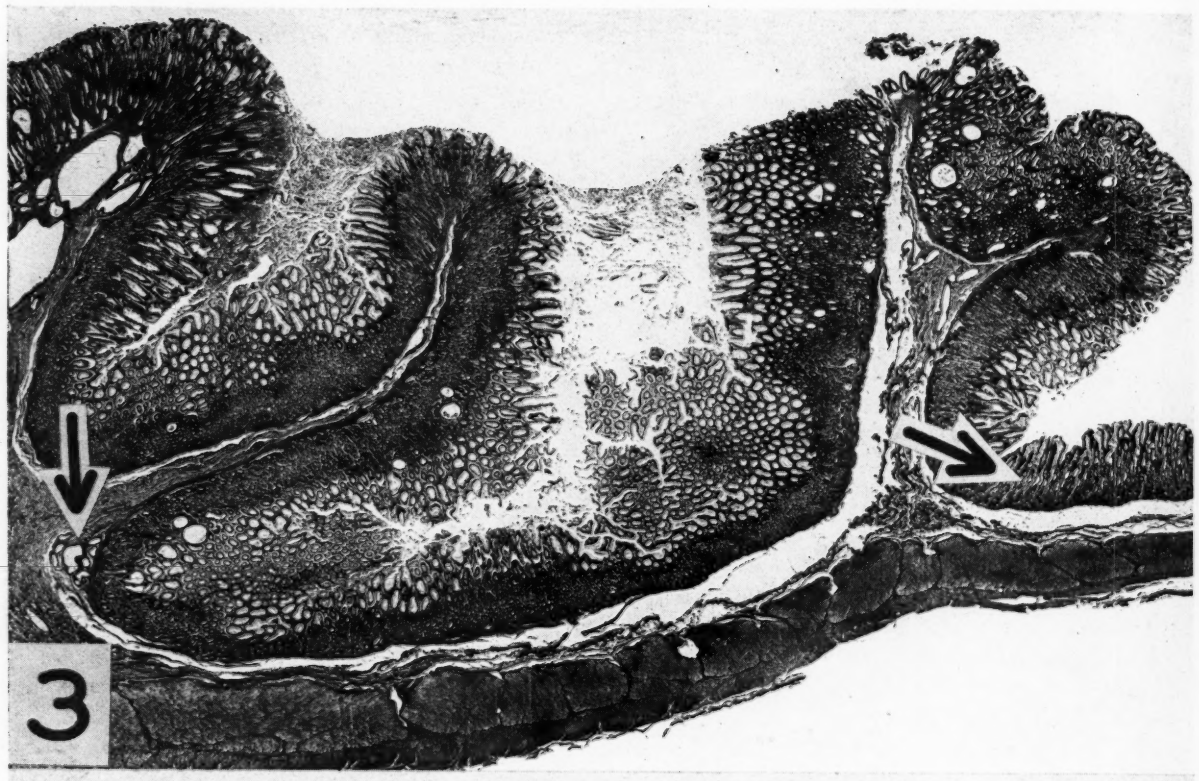


FIG. 3.—Photomicrograph of the fundal border of the lesion shown in Fig. 2, showing the hyperplastic mucosa with early penetration of the submucosa (arrow, left) and normal epithelium (arrow, right). The rugal folds are thickened by the mucosa alone. Mag. ×13.

FIG. 4.—Photomicrograph of the pyloric border of the lesion in Fig. 2, showing the hyperplastic gastric mucosa extending to the duodenum. Mag. ×10.

chiefly of mucus-secreting glands (Fig. 7), numerous minute gastric ulcers, and many foci where the muscularis mucosae was penetrated and the edematous, fibrotic submucosa was invaded by cystic glands (Fig. 9). This lesion was identical with those previously reported (5), and the similarity to giant hypertrophic gastritis of man was quite pronounced (Fig. 8). Oil was found in macrophages in hyperplastic perigastric lymph nodes in this monkey. This animal also had a focal subacute colitis in which the submucosa was invaded by penetrating hyperplastic glands in two small areas. In monkey 3, dead after 165 days of the oil, the entire gastric mucosa was abnormal. There was little submucosal fibrosis and only occasional instances of penetration of the muscularis mucosae by surface epithelium. No parietal or zymogen cells were recognizable. The entire epithelium seemed to be replaced by mucus-secreting cells or poorly differentiated hyperchromatic glands with crowded nuclei. These glands were irregular in size, tortuous, disorderly in arrangement and imbedded in a fibrous lamina propria (Fig. 10). The colon and rectum of this animal were normal. Monkey 4, dead after 175 days, had only partial atrophy of the gastric parietal cells and simplification of the gastric mucosa but the rectum was quite abnormal. Here there were numerous acute superficial ulcerations in the hyperplastic mucosa which was focally heavily infiltrated by polymorphonuclear leukocytes. In many places a few hyperplastic glands invaded the apparently normal submucosa. Monkey 5, dead after 213 days, with stomal gangrene, had a stomach in which the fundus and cardia showed atrophic gastritis. In the cardia, the simplified glands were tortuous and penetrated the muscularis mucosae in numerous areas. The rectal mucosa was extremely hyperplastic and invaded the submucosa extensively. As in the rat lesions with this oil (9) the penetrating glands often contained bacteria and leukocytes or formed abscesses in which the epithelium was necrotic. Large necrotic fissures and diverticula penetrating all the muscular coats of the rectum appeared to be the end result of this process (Figs. 14 and 15).

The effect of protein starvation and ingestion of oil on the gastro-intestinal tract.—Six months of a non-protein diet without the oil did not alter the mucosa of the gastro-intestinal tract of one monkey, but submucosal edema was prominent in the colon of this animal. The monkey that died after 48 days on the non-protein diet containing lubricating oil had an abnormal stomach, cecum, and adjacent colon. There were focal atrophic and hyperplastic mucosal changes in the stomach with a few instances of penetrating glands. In the cecum and colon there were several hemorrhagic, flat, stiff ulcers surrounded by edematous, hyperplastic mucosa. Microscopically these areas were covered by a leukocytic exudate and a low glandular epithelium in which the cells were quite hyperchromatic. Many bottle-shaped deeply penetrating glands were present in the fibrotic submucosa (Fig. 16). In other areas the invading glands were small, irregular in size and shape, and deeply stained with hematoxylin (Fig. 17). The gastro-intestinal tract of the monkey that ate the non-protein diet without oil for 6 months and was then changed to the normal diet for 78 days was normal. The monkey that died after 78 days on the non-protein diet and oil diet had severe hyperplastic gastritis in which almost the entire mucosa was composed of mucus-secreting glands (Fig. 12). These glands contained tall columnar cells except in their deepest portions where the acini were crowded with hyperchromatic, poorly differentiated cells (Fig. 13). In foci the process of replacement of the normal epithelium by the mucus-secreting cells appeared to be still in progress. In such areas the proliferating acini formed boot-shaped glands as they grew against the resistance of the submucosa (Fig. 11).

DISCUSSION

These experiments demonstrate that infiltrating adenomatous lesions of the rhesus monkey can be produced with a high percentage of success by feeding the animals this type of motor lubricating oil. They also show that following the treatment the same type of change occurs in the colon and rectum of this species. The two instances where the gastric submucosa was invaded by cystic mucosal glands in monkeys who were not in contact with the oil indicate that this oil is not the only agent which will produce these changes. Evidently, the gastric and colonic mucosae of the rhesus monkey are extremely reactive to non-specific irritative substances. Starvation alone, and particularly protein-starvation, did not produce these reactive changes. The extremely short induction period for these changes when the animals are fed a non-protein diet along with the oil may be indicative of an accelerating effect of generalized lowered resistance to injury. The microscopic similarity of the many instances of atrophic and hyperplastic gastritis to these conditions in man appear to indicate that the mucosa of the monkey reacts similarly to that of the human stomach and colon. These experiments contrast with those of Pfeiffer and Allen (7) who found extremely high resistance of this species to irritative and carcinogenic agents.

By the usual histological criteria used to diag-

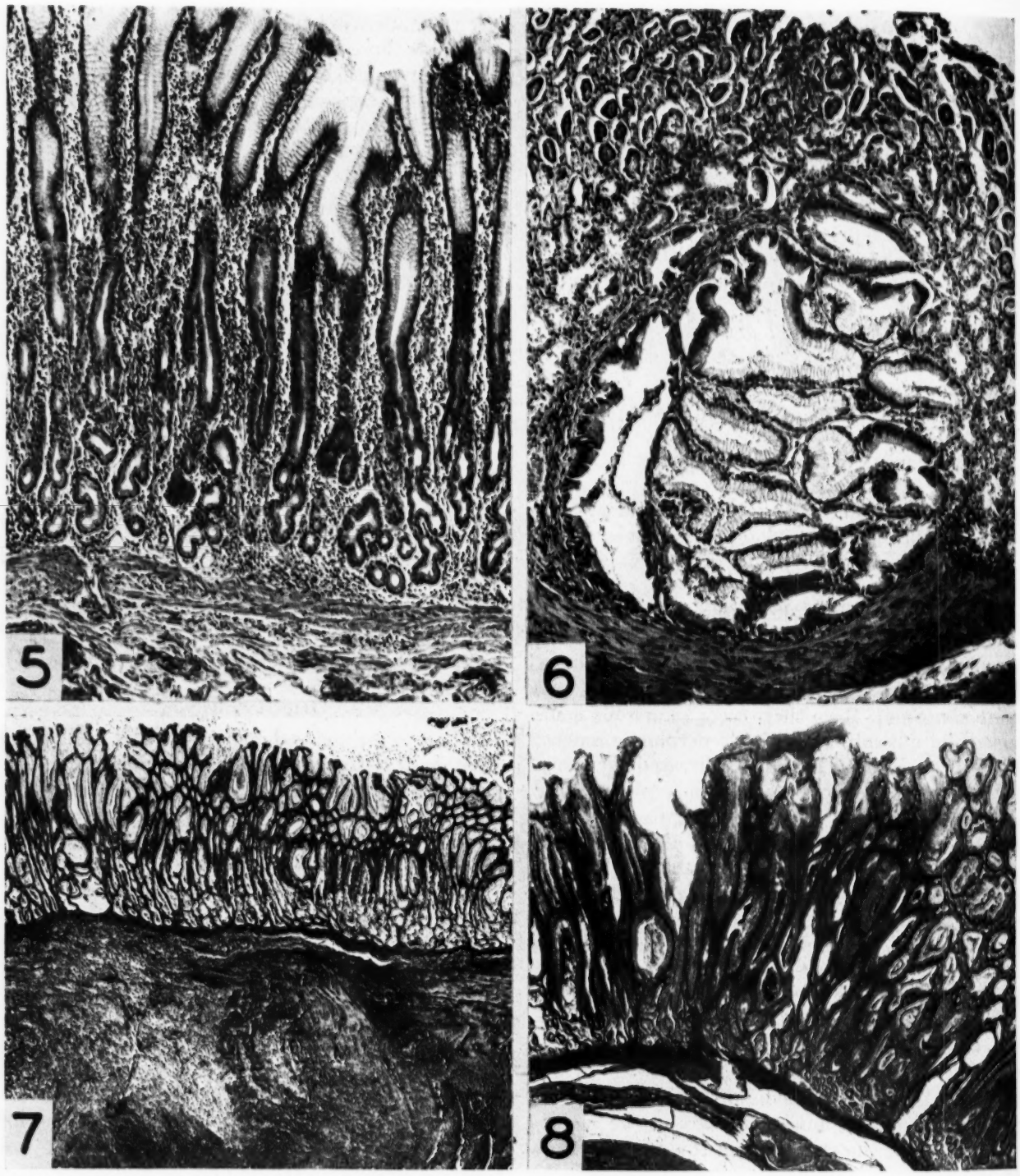


FIG. 5.—Photomicrograph of atrophic gastritis in the fundus of a monkey dead after being sprayed with diesel lubricating oil for 100 days. Mag. reduced from ×120.

FIG. 6.—Photomicrograph of a cystic intramucosal focus of hyperplastic mucus neck glands in the fundus of a monkey that ingested the oil in a synthetic non-protein diet for 48 days. Mag. reduced from ×125.

FIG. 7.—Photomicrograph of the stomach of a monkey sprayed daily for 140 days with the oil, showing hyperplastic simplified epithelium composed predominantly of mucous-secreting cells. Compare with Fig. 8. Mag. reduced from × 30.

FIG. 8.—Photomicrograph of the fundus of a human stomach removed because of giant hypertrophic gastritis. Hematoxylin and phloxin stain. Mag. reduced from ×32.

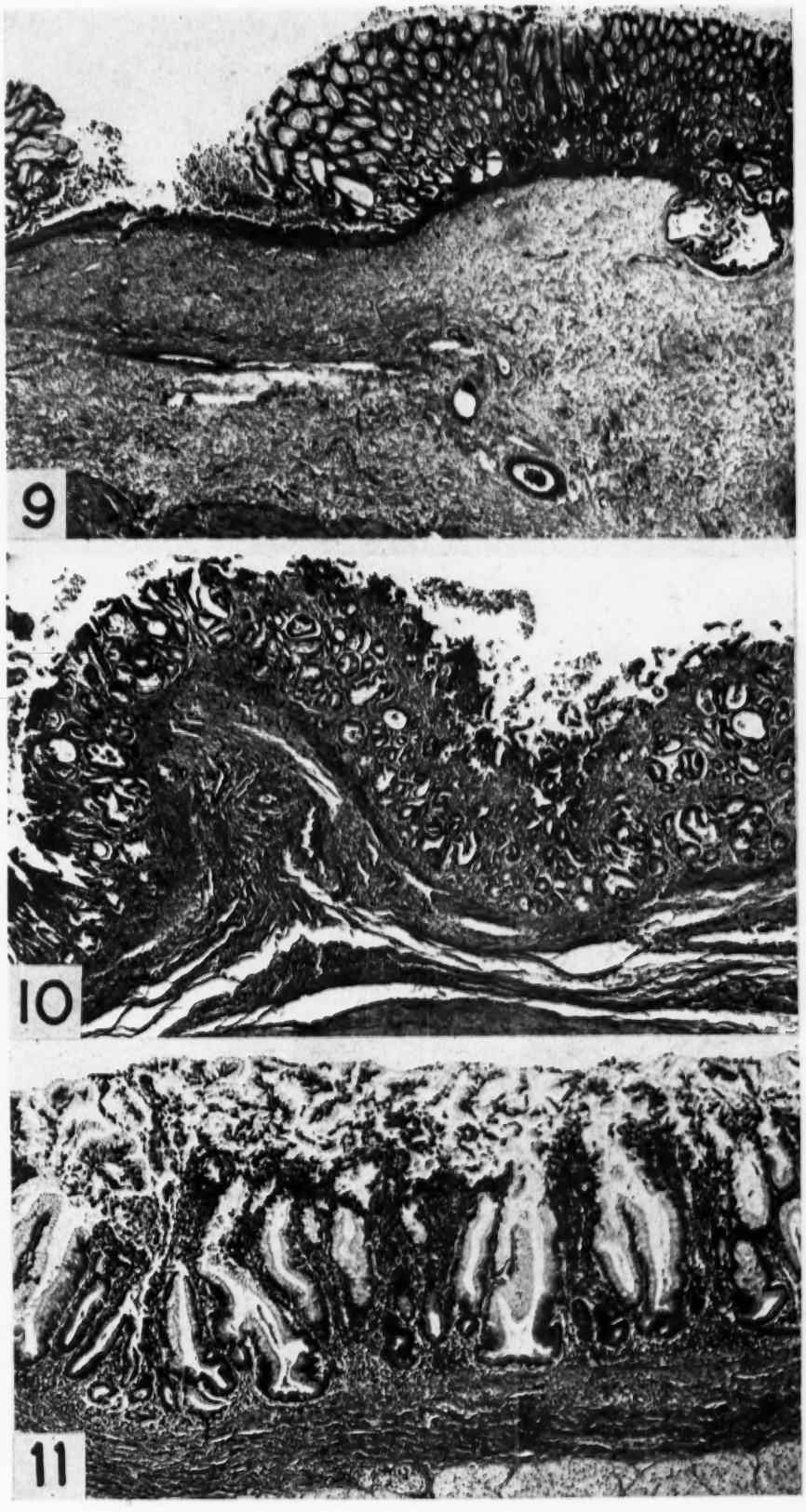


FIG. 9.—Photomicrograph of another area in the stomach of the monkey depicted in Fig. 7, showing the hyperplastic mucosa, an ulcer with partial reepithelialization, penetration of the muscularis mucosae and infiltration of the submucosa by cystic glands, and edema and fibroplasia of the submucosa. Mag. reduced from ×30.

FIG. 10.—Photomicrograph of the gastric mucosa of a monkey sprayed with the oil for 165 days, showing the hyperplastic, hyperchromatic disorderly glands, a lesion compatible in man with carcinoma *in situ*. Mag. reduced from ×56.

FIG. 11.—Photomicrograph of the gastric mucosa of a monkey that ingested the oil in a non-protein diet for 78 days, showing active replacement of the normal epithelium by mucous-secreting glands and the horizontal growth of the abnormal glands along the muscularis mucosae. Mag. reduced from ×82.

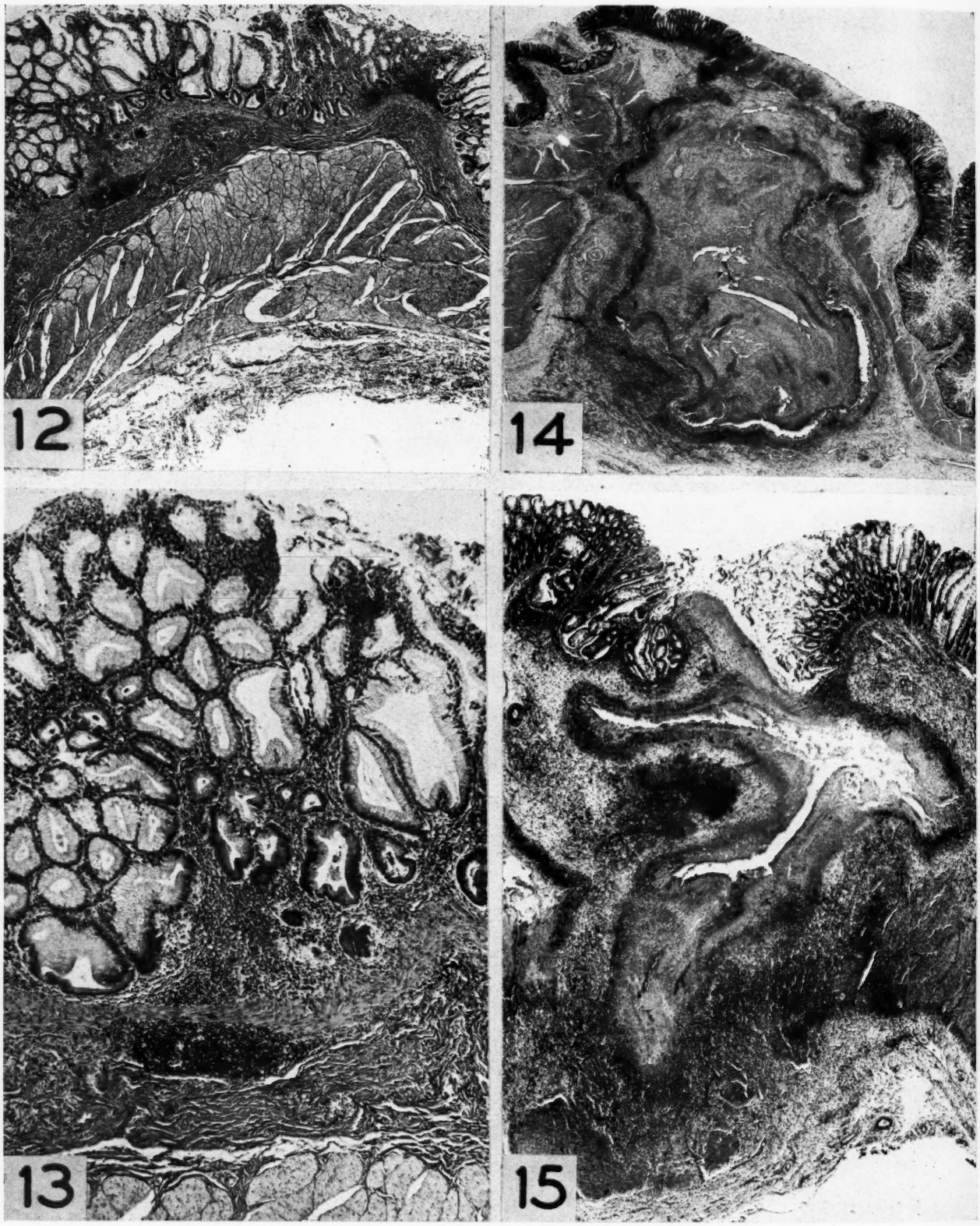
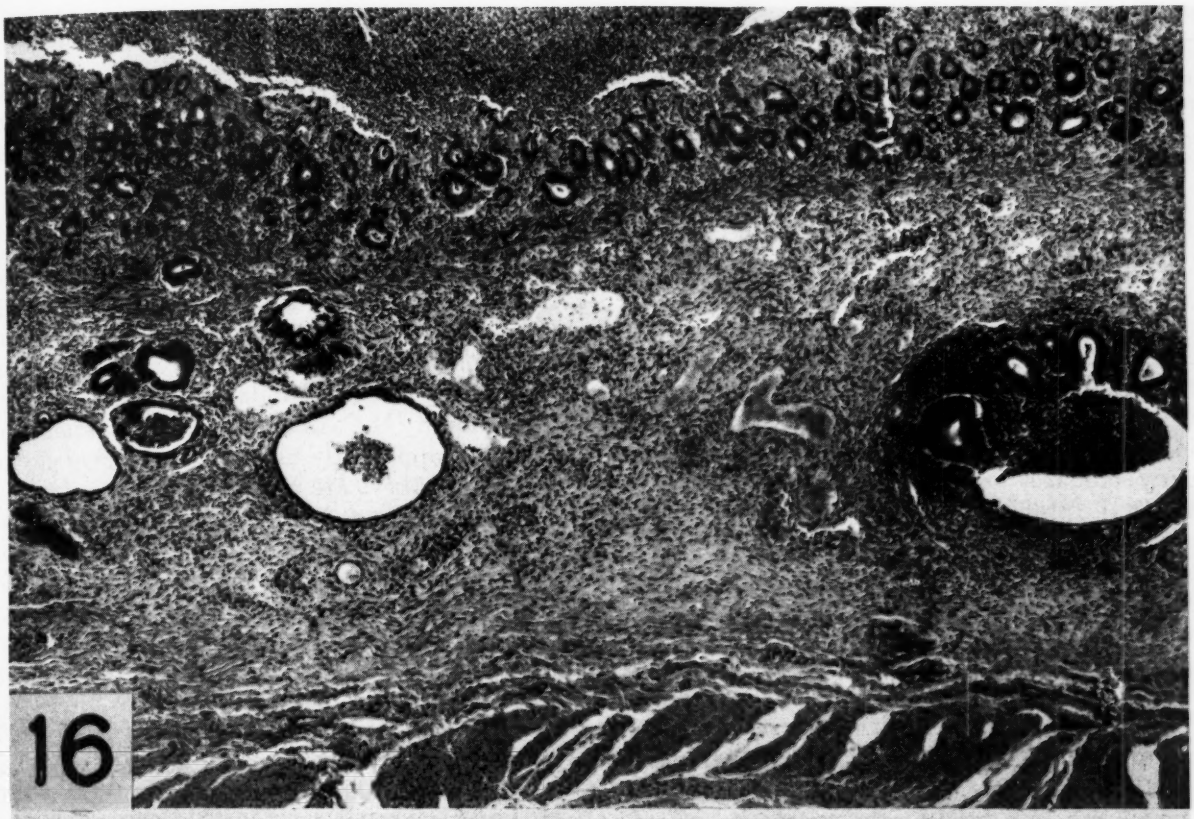


FIG. 12.—Photomicrograph of another area in the stomach in Fig. 11, showing the cystic, hyperplastic, simplified mucosa with chronic inflammation and the change in the crypts to small, poorly differentiated hyperchromatic acini with crowded cells. Mag. reduced from ×20.

FIG. 13.—Photomicrograph of an area in Fig. 12 in greater detail. Mag. reduced from ×82.

FIG. 14.—Photomicrograph of the rectum of a monkey sprayed on the chest with the oil for 213 days, showing a diverticulous abscess penetrating all layers except the subperitoneal connective tissue. Mag. reduced from ×11.

FIG. 15.—Photomicrograph of another area in the rectum in Fig. 14 but nearer the anus, showing another necrotic fissure bordered by hyperchromatic cystic glands that have invaded the submucosa. Mag. reduced from ×30.



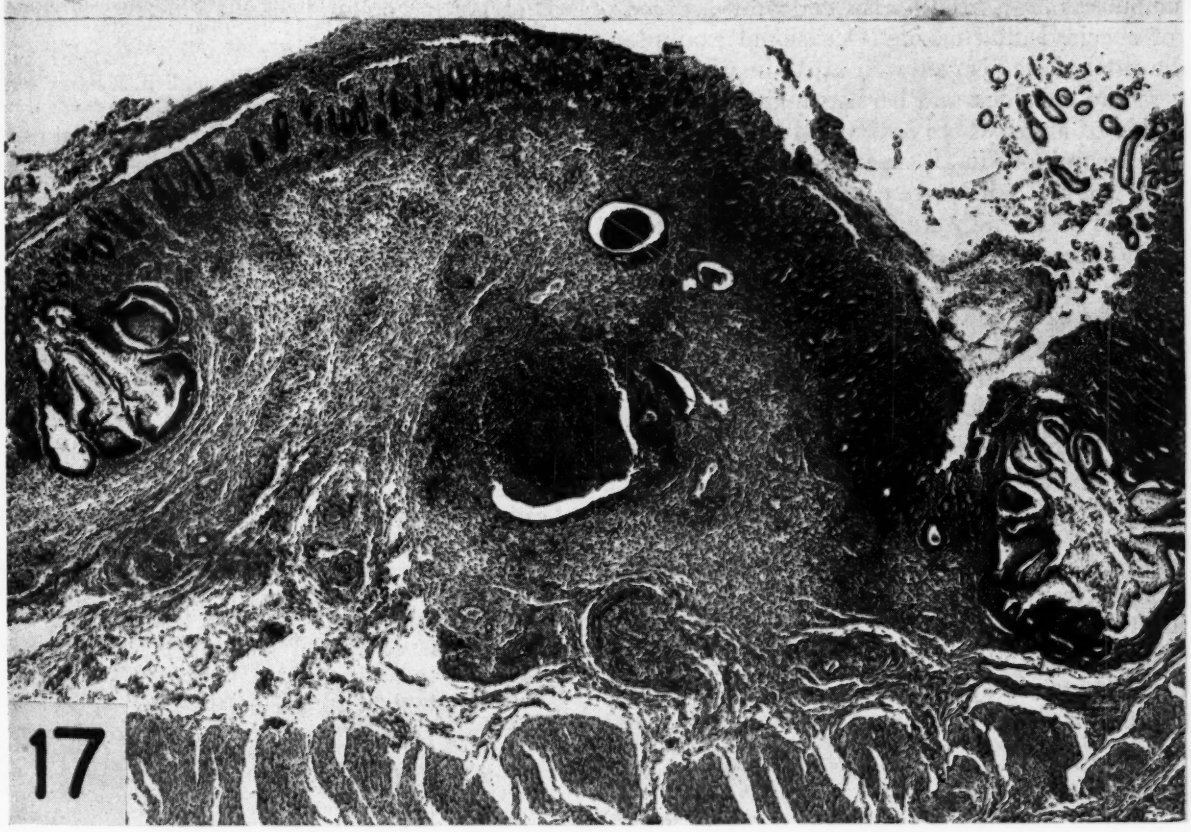


FIG. 16.—Photomicrograph of the cecum of a monkey that ingested the oil in the non-protein diet for 48 days showing an intense cecitis and infiltration of the fibroplastic submucosa by cystic and hyperchromatic glands. Mag. ×56.

FIG. 17.—Photomicrograph of another area in the colon in Fig. 16 showing other examples of invading hyperplastic glands in the fibroplastic submucosa and an early abscess in one of these. Mag. ×30.

nose malignancy many of these lesions would be called cancerous. Heterotopia, hyperchromasia, poor differentiation, and disorderly cell growth are all present. The lesion in monkey 3 (Fig. 10) is morphologically compatible with the diagnosis of gastric carcinoma *in situ*. The lesions in the colon of the monkey fed oil and a non-protein diet for 48 days are by these criteria early invasive carcinomas (Figs. 16 and 17). Experimentally, however, these lesions cannot be classified as malignant neoplasia. Inflammation and infection appear to play as great a role in the production of these lesions as does the oil. Acute and chronic inflammatory exudates are usually present in these lesions. In the previous report (5), one monkey, that clinically was suffering from the same diseases as the others that died with infiltrating hyperplastic adenoma-like lesions of the stomach, was saved through diligent nursing care. It was killed about one and a half years later. At that time only a severe atrophic gastritis was present in this animal. In the experiments with rats (9) in which many of the lesions appeared just as malignant as these, the production of the lesions in the presence of the oil was prevented by the concomitant feeding of succinylsulfathiazole. Occasional examples of the rat lesions also showed areas in which the process had subsided and healing had taken place alongside of areas where active lesions were present. In the animals in all experiments with the oil, regional lymph nodes were carefully examined for metastases and none were found. In the monkeys the glands never reached or invaded the external muscular coats of the stomach or intestine. Fibroplasia in the submucosa was abundant and always resulted in a layer of fibrous tissue between the growing glands and the muscle. The extremely short induction period for these lesions, varying between 48 and 213 days, also speaks against these lesions being neoplastic.

The pathogenesis of these lesions in the monkey cannot be reconstructed from this limited material. The pathogenesis was discussed in the case of the colonic lesions in the rat (9). Because of the similarity of these lesions to many human mucosal abnormalities an understanding of the mechanism of their production and fate would be useful in human pathology. An experiment is now in progress in which an attempt is being made to diagnose the presence of such lesions in the monkey before the animals become moribund so that the progression and fate of the lesions can be learned.

SUMMARY

Previous observations on the occurrence of infiltrating hyperplastic gastric mucosal lesions in rhesus monkeys who had ingested diesel motor lubricating oil are extended by additional examples of the disease. Similar lesions are produced in the colon by the same means. Two instances of similar but apparently naturally occurring gastric lesions in monkeys are reported. These lesions are described and their nature is discussed. Their similarity to human disease and, in several instances to human gastric and colonic carcinoma, are pointed out. These lesions are considered in the absence of incontrovertible proof not to be malignant neoplasms in spite of their morphological appearance.

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A Lymphopenia-Causing Agent, Probably a Virus, Found in Mice After Injection with Tumor Tissue and with Cell-free Filtrates of Lymphosarcoma T 86157 (MB)

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In a previous hematological study (1) of mice which had been injected subcutaneously with minced tumor tissue of lymphosarcoma T 86157 (MB) in Tyrode's solution, it was found that a leukopenia appeared on the second day after inoculation. This decrease of leukocytes represented a true lymphopenia, which disappeared a few days afterwards. The question arose whether this lymphopenia was caused by the tumor, or whether it had some other excitant. Various substances were therefore injected into mice, to investigate whether they would produce the same phenomenon as the MB tumor did. Also attempts were made to separate the tumor-inducing agent from the substance which caused the lymphopenia, by injections of cell-free filtrates and by injections of tumor into mice of other strains than the F_1Bd[1] mice.

EXPERIMENTS

In Tables 1 through 5 some of the results are shown. In Table 1 the blood counts of four normal F_1Bd mice are presented as controls. In the other four tables the blood counts of mice 48 hours after injection are given.

The tumors and organs were minced with scissors in Tyrode's solution, and 0.2 cc. of this preparation was injected subcutaneously into F_1Bd and other mice.

Experiment 1.—(Table 2). The following materials were each injected into two F_1Bd mice: (a) Tyrode's solution; (b) spleen and liver from a normal F_1Bd mouse; (c) spleen and liver from a normal B mouse. The blood picture of all the mice injected was normal 48 hours after injection.

TABLE 1

TOTAL AND DIFFERENTIATED WHITE BLOOD COUNTS IN NORMAL F_1Bd MICE

		Mouse number			
		A	B	C	D
Eosinophils	—number	0	0	90	0
	—per cent	0	0	3	0
Stab-cells	—number	0	23	0	0
	—per cent	0	½	0	0
Polymorphonuclears	—number	1260	846	660	1073
	—per cent	28	18	22	29
Lymphocytes	—number	3015	3619	2130	2627
	—per cent	67	77	71	71
Monocytes	—number	225	211	90	0
	—per cent	5	4½	3	0
Unclassified cells	—number	0	0	30	0
	—per cent	0	0	1	0
Totals		4500	4700	3000	3700

TABLE 2

TOTAL AND DIFFERENTIAL WHITE BLOOD COUNTS IN F_1Bd MICE INJECTED WITH DIFFERENT MATERIALS

		Injected material							
		Tyrode's solution		Organs from F_1Bd mouse		Organs from B mouse		Tumor round-cell sarcoma MA	
Mouse number		1	2	3	4	5	6	7	8
Eosinophils	—number	31	0	39	0	0	75	0	39
	—per cent	½	0	½	0	0	1½	0	1½
Stab-cells	—number	31	0	0	0	19	25	32	0
	—per cent	½	0	0	0	½	½	1	0
Polymorphonuclears	—number	1147	720	1092	1100	722	900	800	598
	—per cent	18½	22½	14	22	19	18	25	23
Lymphocytes	—number	4929	2416	6591	3700	2983	3775	2340	1963
	—per cent	79½	75½	84½	74	78½	77½	72	75½
Monocytes	—number	31	64	78	150	57	50	64	0
	—per cent	½	2	1	3	1½	1	2	0
Unclassified cells	—number	31	0	0	50	19	75	0	0
	—per cent	½	0	0	1	½	1½	0	0
Totals		6200	3200	7800	5000	3800	5000	3200	2600

[1] B = C 57 black Little. d = dilute brown Murray-Little. F_1Bd = F_1B ♀ × d♂.

Experiment 2.—(Table 2). To investigate whether another round-cell sarcoma would produce a similar lymphopenia, 2 mice were injected with round-cell sarcoma T 90904 (MA) which is not related to tumor MB. No lymphopenia could be demonstrated 2 days after injection.

It seemed then that producing a decrease in lymphocytes was a specific function of round-cell sarcoma MB.

TABLE 3

TOTAL AND DIFFERENTIAL WHITE BLOOD COUNTS IN F_1Bd MOUSE NO. 10

INJECTED MATERIAL		1ST INJECTION Filtrate of tumor MB	2D INJECTION Filtrate of tumor MB	3D INJECTION Minced tumor MB
Eosinophils	—number	11	0	68
	—per cent	1	0	2
Metamyelocytes	—number	0	56	34
	—per cent	0	½	2
Stab-cells	—number	0	0	34
	—per cent	0	0	1
Polymorphonuclears	—number	748	3192	663
	—per cent	68	28½	19½
Lymphocytes	—number	275	7840	2516
	—per cent	25	70	74
Monocytes	—number	66	112	34
	—per cent	6	1	1
Unclassified cells	—number	0	0	34
	—per cent	0	0	1
Totals		1100	11200	3400

c) Five mice which had been once or twice injected with cell-free filtrates were inoculated subcutaneously with tumor. No lymphopenia was observed, although the growth of the tumor was comparable with that developed in mice not previously injected with cell-free filtrate. It is evident that after injection with a cell-free filtrate of the tumor some immunity was built up relative to the lymphopenia-causing agent but that the growth of the tumor was not affected. The lymphopenia-causing "agent" passed a size 3 Seitz filter, whereas the tumor-producing "agent" did not.

Experiment 4.—(Table 4).

a) Minced liver and spleen from mice previously injected with cell-free filtrates were inoculated into 6 mice. Mice Nos. 81 and 82 are used to illustrate this experiment. All 6 mice developed a lymphopenia on the second day after injection. Liver and spleen from these 6 mice again produced lymphopenia when injected into other mice (Nos. 129 and 130). Minced organs from mouse No. 130 produced lymphopenia when injected into 2 mice (Nos. 236 and 239).

b) Heart blood taken from a mouse which had been injected with cell-free filtrate of tumor MB was injected into two mice, which developed lymphopenia after 48 hours.

A lymphopenia-producing "agent" was demonstrated in the spleen, liver, and blood of mice by serial passages of these tissues. The first two mice in this series had been injected with cell-free filtrate of tumor MB. Some of the mice were not killed but no sequelae were seen to develop.

TABLE 4

TOTAL AND DIFFERENTIAL WHITE BLOOD COUNTS IN F_1Bd MICE INJECTED WITH MINCED ORGANS IN TYRODE'S SOLUTION

		INJECTED MATERIAL							
		Organs from normal F_1Bd mouse		Organs from F_1Bd mouse injected with filtrate from tumor MB		Organs from F_1Bd mouse 81		Organs from F_1Bd mouse 130	
Mouse number		3	4	81	82	129	130	236	239
Eosinophils	—number	39	0	12	28	140	38	45	54
	—per cent	½	0	1	4	5	2	3	6
Stab-cells	—number	0	0	0	7	0	0	0	9
	—per cent	0	0	0	1	0	0	0	1
Polymorphonuclears	—number	1092	1100	756	469	1484	1349	795	396
	—per cent	14	22	63	67	53	71	53	44
Lymphocytes	—number	6591	3700	384	182	756	494	555	297
	—per cent	84½	74	32	26	27	26	37	33
Monocytes	—number	78	150	48	7	420	19	105	144
	—per cent	1	3	4	1	15	1	7	16
Totals		7800	5000	1200	700	2800	1900	1500	900

It was demonstrated by additional experiments, however, that this was not the case.

Experiment 3.—(Table 3).

a) Ten F_1Bd mice were injected with a cell-free filtrate of tumor MB. All the mice showed a strong lymphopenia on the second day after injection. No tumors were produced, however. Mouse No. 10 is used to illustrate this experiment.

b) Eight of the mice mentioned under experiment 3a were injected for a second time with cell-free filtrate of tumor MB. No lymphopenia could be demonstrated.

Experiment 5.—(Table 5).

a) Minced tumor MB was injected into four 0 20, two d, and four B mice. In the 0 20 and in the B mice a tumor was produced which disappeared. In the d mice no tumors were produced. All mice developed lymphopenia. No lymphopenia was observed in the control animals of the same strains. The lymphopenia in the strains not susceptible to tumor MB was less conspicuous than that which occurred in the F_1Bd mice.

b) After a second inoculation with tumor MB into the mice of experiment 5a no lymphopenia developed.

c) A cell-free filtrate of the tumor MB produced lymphopenia after the first injection, but none after a second injection.

d) Minced liver and spleen derived from B mouse No. 58 (previously injected with tumor MB) caused lymphopenia when injected into two F_1Bd mice, Nos. 93 and 94 (Table 6). After four serial liver and spleen transferals in F_1Bd mice the lymphopenia could still be demonstrated (mice Nos. 289 and 290).

Experiment 6.—Tumor MB was cultvated *in vitro* as previously described (1). Cultures containing typical lymphoblast-like cells produced a tumor when injected into F_1Bd mice.

a) Six mice were inoculated with cultures of lymphosarcoma MB. No lymphopenia developed, whereas the tumor growth was unaffected.

b) A new strain of MB tumor was established in mice injected with cells of sarcoma MB cultivated *in vitro*. This strain maintained its characteristics when transplanted into F_1Bd mice and even after 34 passages in the mouse did not produce a lymphopenia. Grossly this new tumor did not differ from the original tumor MB. The blood picture of mice bearing the new tumor was the same as that of mice injected with the original tumor MB, except for the absence of a lymphopenia on the second day after inoculation. This result is given as further evidence that the presence of the tumor is not directly related to the production of the lymphopenia observed.

SUMMARY

1. A lymphopenia was observed to develop in mice on the second day following injection of minced tumor tissue of lymphosarcoma T 86157 (MB).

2. It was shown that a tumor could be produced in mice without a concomitant lymphopenia.

3. Lymphopenia without tumor was produced in mice injected with cell-free filtrate of sarcoma MB.

4. Lymphopenia without tumor was also produced by injection of minced organs derived from mice previously injected with cell-free filtrate of sarcoma MB.

5. It is concluded that a filtrable agent, probably a virus, is responsible for the lymphopenia produced. This virus seems to bear no relationship to the presence of the tumor.

TABLE 5

TOTAL AND DIFFERENTIAL WHITE BLOOD COUNTS IN MICE OF VARIOUS STRAINS, 48 HOURS AFTER INJECTION WITH MINCED TUMOR MB, AND IN CONTROL MICE OF THE SAME STRAINS

Mice	Treatment	Totals	Lymphocytes (in per cent)	Polymorphonuclears (in per cent)
0 20 mice—	non-injected			
No. 155		6000	64	30
156		7800	61	29
175		2600	73	22
176		2500	73	13
0 20 mice—	injected			
No. 153		3200	48	50
154		2000	60	39
173		1500	55	39
174		1500	52	44
d mice—	non-injected			
No. 185		4800	55	45
186		4100	67	32
d mice—	injected			
No. 183		1900	35	59
184		3400	33	67
B mice—	injected			
No. 58		1600	33	62
59		1500	39	60
60		1200	38	48
61		1100	33	55

TABLE 6

TOTAL AND DIFFERENTIAL BLOOD COUNTS IN B AND F_1Bd MICE INJECTED WITH DIFFERENT MATERIALS

Mouse	Injected material	Total count	Eosinophils (in per cent)	Polymorphonuclears (in per cent)	Lymphocytes (in per cent)	Monocytes (in per cent)
Normal F_1Bd	Non-injected	±4000	±½	±24	±71	± 3
B No. 58	Tumor MB	1600	3	62	33	2
B No. 59		1500	0	60	39	1
F_1Bd No. 93	Organs from	1400	7	47	45	1
F_1Bd No. 94	mouse No. 58	800	2	69	23	6
F_1Bd No. 136	Organs from	1000	2	57	37	4
F_1Bd No. 137	mouse No. 93	1100	2	67	26	5
F_1Bd No. 240	Organs from	1000	0	39	57	4
F_1Bd No. 241	mouse No. 137	700	1	64	32	3
F_1Bd No. 289	Organs from	1400	3	55	36	6
F_1Bd No. 290	mouse No. 240	2100	3	38	41	18

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Studies on the Intracellular Composition of Livers from Rats Fed Various Aminoazo Dyes*

I. 4-Aminoazobenzene, 4-Dimethylaminoazobenzene, 4′-Methyl-, and 3′-Methyl-4-Dimethylaminoazobenzene

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When the hepatic carcinogen 4-dimethylaminoazobenzene is fed to rats the intracellular composition of the liver is altered considerably (6, 7). After four weeks there is an increase in the desoxypentosenucleic acid and protein contents of the nuclear fraction, and a marked decrease in the amounts of protein, riboflavin, and pentosenucleic acid in the large granules (mitochondria). A decrease in the pentosenucleic acid level also occurs in the small granules (microsomes), and protein-bound aminoazo dye is found in each fraction, with the highest concentration occurring in the supernatant fluid (particles not sedimented at 19,000 × g). These alterations in intracellular composition are even more exaggerated in the hepatic tumors induced by the dye (7).

If any of these changes are related to the carcinogenic activity of the dye, one might expect that other aminoazo dyes which are either more or less active than 4-dimethylaminoazobenzene would produce similar changes to a greater or lesser extent, respectively. On the other hand, if the changes are only manifestations of the general toxic properties of aminoazo dyes, one would expect little or no correlation between carcinogenic potency and the observed changes in intracellular composition. In the present study three aminoazo dyes closely related to 4-dimethylaminoazobenzene were fed to rats and their effects on the intracellular composition of the liver were compared. The three dyes selected, 4-aminoazobenzene, 4′-methyl-4-dimethylaminoazobenzene, and 3′-methyl-4-dimethylaminoazobenzene, have carcinogenic activities of 0, less than 1, and 10 to 12, respectively, as compared with the arbitrarily chosen activity of 6 for the reference compound 4-dimethylaminoazobenzene (5).

METHODS

These experiments were carried out in two separate but identical series. For each series 5 groups of male rats[1] weighing 180 to 200 gm. were fed *ad libitum* a semi-synthetic diet (4, diet 3) containing 1.2 mgm. of riboflavin per kgm. One group was fed the basal diet (no dye added) while the others were fed diets containing 0.06 per cent of 4-dimethylaminoazobenzene or equimolar levels of one of the other dyes. Four weeks after the rats were placed on the experimental diets the group receiving 3′-methyl-4-dimethylaminoazobenzene was killed, and the remaining groups were killed at 2 day intervals in the order of decreasing carcinogenicities of the dyes fed.

For each fractionation the livers of 3 rats were perfused *in situ*, forced through a plastic tissue mincer, and homogenized in 0.88 *M* sucrose as previously described (6, 7). The fractionation procedure was the one used previously (6, 7) except that the large and small granules were sedimented in an improved rotor[2] (designed in collaboration

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[1] Obtained from the Holtzman Rat Company, Madison, Wisconsin.

[2] This rotor fits the spindle on the high speed attachment sold by the International Equipment Co., Boston, Mass. It is made of a magnesium alloy and has an outside diameter of $6\frac{1}{2}$ inches and an over-all height of $4\frac{3}{8}$ inches. It holds twelve $\frac{5}{8}$ inch × $3\frac{3}{8}$ inches plastic tubes, each with a capacity of 13 ml., at an angle of 25° from vertical. The distance from the axis to the geometrical center of the fluid is 6.5 cm. when the tube is filled and the rotor is revolving. It was made by Joseph Grebmeier and Sons, P.O. Box 235, Menlo Park, California.

with Dr. W. C. Schneider). The large granules were still sedimented by applying a force of 19,000 × g for 10 minutes, but since a centrifugal force of 25,000 × g was obtained at the center of the tubes at full speed, 3 hours at the higher centrifugal force were found to be sufficient to sediment the small granules.

Each fraction and the whole homogenate were analyzed for protein, desoxypentosenucleic acid, pentosenucleic acid, riboflavin, and protein-bound

clear protein, but the protein content of this fraction increased by 133 per cent when the more active carcinogen 3′-methyl-4-dimethylaminoazobenzene was fed. The large and small granule fractions from the livers of rats fed 4-dimethylaminoazobenzene contained 32 and 18 per cent less protein than the same fractions of the control livers; even larger decreases of 57 and 24 per cent, respectively, were found when the 3′-methyl dye was fed.

TABLE 1

DISTRIBUTION OF PROTEIN IN THE LIVER FRACTIONS*
(Azo Dye Fed)

FRACTION	NONE	AB†	4′-ME-DAB†	DAB	3′-ME-DAB
		Milligrams of protein per gram of fresh liver‡			
Whole homogenate	121–123	130–118	111–115	107–106	111–120
Nuclei	15– 16	12– 17	14– 16	18– 16	36– 37
Large granules	39– 41	45– 35	38– 34	26– 29	15– 20
Small granules	16– 18	20– 18	17– 18	12– 16	12– 14
Supernatant fluid	47– 47	51– 46	42– 45	45– 41	41– 49
Recovery	117–122	128–116	111–113	101–102	104–120

* The first and second numbers in each column refer to the first and second series of experiments, respectively, as mentioned in the text.

† AB = 4-aminoazobenzene.
DAB = 4-dimethylaminoazobenzene.

‡ The figures to the nearest whole numbers.

TABLE 2

DISTRIBUTION OF DESOXYPENTOSENUCLEIC ACID IN THE LIVER FRACTIONS*
(Azo Dye Fed)

FRACTION	NONE	AB†	4′-ME-DAB†	DAB	3′-ME-DAB
		Milligrams of nucleic acid per gram of fresh liver			
Whole homogenate	1.78–1.94	1.79–1.93	1.95–1.75	2.28–2.10	4.78–4.22
Nuclei	1.84–1.89	1.82–1.93	1.85–1.86	2.44–2.01	4.54–3.95
Recovery	1.84–1.89	1.82–1.93	1.85–1.86	2.44–2.01	4.54–3.95
		Milligrams of nucleic acid per gram of protein‡			
Whole homogenate	15– 16	14– 16	18– 15	21– 20	43– 35
Nuclei	123–118	152–113	132–116	135–126	126–107

* The first and second numbers in each column refer to the first and second series of experiments, respectively, as mentioned in the text.

† AB = 4-aminoazobenzene.
DAB = 4-dimethylaminoazobenzene.

‡ The figures to the nearest whole numbers.

aminoazo dye as described previously (6), and the average recoveries of these substances in the fractions were 98, 100, 95, 102, and 98 per cent, respectively, of the amounts found in the whole homogenates.

RESULTS

Protein distribution.—The intracellular distribution of protein in the livers of the rats fed the basal diet was similar to that observed previously (6), and the ingestion of either 4-aminoazobenzene or 4′-methyl-4-dimethylaminoazobenzene did not alter the distribution significantly (Table 1). In these series ingestion of 4-dimethylaminoazobenzene caused little or no increase in the level of nu-

Desoxypentosenucleic acid distribution.—Regardless of the dye fed, the desoxypentosenucleic acid was always found only in the nuclear fraction (Table 2). Neither the ingestion of 4-aminoazobenzene nor 4′-methyl-4-dimethylaminoazobenzene altered the level of this nucleic acid in the nuclear fraction, but the ingestion of 4-dimethylaminoazobenzene caused a slight increase (6) while consumption of 3′-methyl-4-dimethylaminoazobenzene resulted in increases of 169 and 117 per cent in the two series. Similar increased levels of desoxypentosenucleic acid in the livers of rats fed the 3′-methyl dye have been observed by Griffin, Nye, Noda, and Luck (1). In spite of the wide differences between the desoxypentosenucleic acid

contents of the nuclear fractions from the livers of rats fed the various dyes, the level of this nucleic acid per gram of nuclear protein was remarkably constant.

Pentosenucleic acid distribution.—In general, ingestion of any of these dyes resulted in reduced levels of pentosenucleic acid in the large and small granules, but the largest differences were found when the more carcinogenic dyes were fed (Table 3). Thus in the two series the average decreases were 12, 23, 33, and 58 per cent for the large granules and 10, 24, 44, and 43 per cent for the small granules from the livers of rats fed 4-aminoazobenzene, 4′-methyl-4-dimethylaminoazobenzene, 4-dimethylaminoazobenzene, and 3′-methyl-4-dimethylaminoazobenzene, respectively. The only other consistent alterations in the distribution of this nucleic acid were average increases of 144 and 32 per cent, respectively, in the amounts found in the nuclear and supernatant fluid fractions from the livers of the rats fed 3′-methyl-4-dimethylaminoazobenzene. In the cases of the nuclear and large granule fractions the protein and pentosenucleic acid levels were altered to about the same extent, so that in spite of large absolute changes the concentrations of this nucleic acid per gm. of protein were unaltered.

TABLE 3

DISTRIBUTION OF PENTOSENUCLEIC ACID IN THE LIVER FRACTIONS*
(Azo Dye Fed)

FRACTION	NONE	AB†	4′-ME-DAB†	DAB	3′-ME-DAB
		Milligrams of nucleic acid per gram of fresh liver			
Whole homogenate	5.32–5.70	5.29–4.68	4.09–4.60	4.27–3.57	5.32–4.81
Nuclei	0.58–0.49	0.34–0.49	0.28–0.43	0.47–0.25	1.58–1.06
Large granules	1.74–1.80	1.88–1.24	1.41–1.33	1.31–1.06	0.75–0.76
Small granules	1.78–1.78	1.70–1.52	1.20–1.43	1.04–0.97	1.01–1.04
Supernatant fluid	1.33–1.11	1.38–1.09	1.06–1.08	1.30–0.96	1.72–1.50
Recovery	5.36–5.18	5.30–4.34	3.95–4.27	4.12–3.24	5.06–4.36
		Milligrams of nucleic acid per gram of protein‡			
Whole homogenate	44–46	41–40	37–40	40–34	48–40
Nuclei	39–31	28–29	20–27	26–16	44–29
Large granules	45–44	42–35	37–39	50–37	50–38
Small granules	111–99	85–85	71–79	87–61	84–74
Supernatant fluid	28–24	27–24	25–24	29–23	42–31

* The first and second numbers in each column refer to the first and second series of experiments, respectively, as mentioned in the text.

† AB = 4-aminoazobenzene.
DAB = 4-dimethylaminoazobenzene.

‡ The figures to the nearest whole numbers.

TABLE 4

DISTRIBUTION OF RIBOFLAVIN IN THE LIVER FRACTIONS*
(Azo Dye Fed)

FRACTION	NONE	AB†	4′-ME-DAB†	DAB	3′-ME-DAB
		Micrograms of riboflavin per gram of fresh liver			
Whole homogenate	9.9– 9.8	9.8–10.0	9.7–14.4	6.5–7.1	7.0–5.5
Nuclei	0.7– 0.5	0.6– 1.0	1.0– 1.2	0.8–0.6	2.0–1.8
Large granules	5.8– 5.1	4.8– 5.5	5.1– 6.5	3.6–3.9	2.1–1.8
Small granules	1.0– 1.6	1.0– 1.7	1.5–2.2	0.8–1.8	0.9–0.6
Supernatant fluid	1.7– 3.5	2.8– 1.6	2.3– 4.0	1.7–1.4	2.4–1.6
Recovery	9.2–10.7	9.2– 9.8	9.9–13.9	6.9–7.7	7.4–5.8
		Micrograms of riboflavin per gram of protein‡			
Whole homogenate	82– 80	75– 85	87–125	61– 67	63–46
Nuclei	47– 31	50– 59	71– 75	44– 38	56–49
Large granules	149–124	107–157	134–191	139–134	140–90
Small granules	63– 89	50– 94	88–122	67–112	75–43
Supernatant fluid	36– 74	55– 35	55– 89	38– 34	59–33

* The first and second numbers in each column refer to the first and second series of experiments, respectively, as mentioned in the text.

† AB = 4-aminoazobenzene.
DAB = 4-dimethylaminoazobenzene.

‡ The figures to the nearest whole numbers.

Riboflavin distribution.—While administration of either 4-aminoazobenzene or 4′-methyl-4-dimethylaminoazobenzene had no consistent effect on the level of riboflavin in any of the fractions, ingestion of either 4-dimethylaminoazobenzene or

its 3′-methyl derivative resulted in decreases of 35 and 65 per cent, respectively, in the level of this vitamin in the large granule fraction (Table 4). The 3′-methyl dye also caused a large increase in the amount of riboflavin in the nuclear fraction. Since in both of these fractions there were approximately proportionate changes in both the riboflavin and protein contents, the concentrations of riboflavin per gm. of protein were not altered.

Distribution of protein-bound dye.—The distribution of protein-bound dye in the livers of rats fed 4-dimethylaminoazobenzene and its 3′-methyl and 4′-methyl derivatives is given in Table 5. In each case 50 to 60 per cent of the bound dye was found in the supernatant fluid fraction, and the proteins of the supernatant fluid and small granules had the highest and second highest concentrations of bound dye, respectively. As observed earlier (2) the bound dye derived from 4-aminoazobenzene absorbed so little light that the necessary correction for non-specific absorption accounted for 30 to 80 per cent of the light absorbed by the extracts from the various fractions. Further, while the bound dyes liberated from the protein contain only 5 to 10 per cent of non-polar dyes in the case of the dimethylamino compounds (2, 3), about 50 per cent of the bound dye derived from 4-aminoazobenzene is non-polar and appears to be 4-aminoazobenzene (2). For these reasons the bound dye analyses on the livers from rats fed the non-methylated dye have not been included in the table.

It is apparent from the table that even the relatively inactive dye 4′-methyl-4-dimethylaminoazobenzene yielded a high level of protein-bound dye. Other studies have shown that, for the dyes studied, the level of bound dye rises to a maximum level and thereafter plateaus or, more often, declines even though the rats continue to ingest dye (3). The rapidity with which the level of bound dye reaches a peak and begins to decline can be correlated with the activities of the dyes. Thus, the maximum levels of bound dye are found in approximately 2, 4, and >16 weeks when 3′-methyl-4-dimethylaminoazobenzene, 4-dimethylaminoazobenzene, and 4′-methyl-4-dimethylaminoazobenzene are fed.

DISCUSSION

Our previous work (6, 7) has shown that 4-dimethylaminoazobenzene induces gross changes in the intracellular composition of the rat liver which are continued in an exacerbated form in the neoplastic state. These changes include increases in the amounts of both nucleic acids and of protein in the nuclear fraction, marked decreases in the amounts of protein, pentosenucleic acid, and riboflavin in the large granules, and a fall in the pentosenucleic acid content of the small granules. In the present study the non-carcinogenic dye, 4-aminoazobenzene, and the weak carcinogen, 4′-methyl-4-dimethylaminoazobenzene, did not significantly alter the levels of desoxypentosenucleic acid, protein, or riboflavin in the cell fractions. However, these two dyes did produce moderate decreases in the level of pentosenucleic acid in the large and small granules. The ingestion of the strong carcinogen, 3′-methyl-4-dimethylaminoazobenzene, produced very marked changes in the liver. The levels of both nucleic acids and of protein in the nuclear fraction increased greatly and approached to a considerable degree the levels previously found in the same fraction of liver tumor (7). Furthermore, the levels of pentosenucleic acid, protein, and riboflavin in the large and small granule fractions of these livers were lowered nearly to the levels found in liver tumors (7). While the increase in the level of pentosenucleic acid in the supernatant fluid produced by the highly carcinogenic 3′-methyl derivative was only half of that found in hepatic tumor tissue (7), this is the first dye which has been found to induce this particular change. It is of interest that neither this dye nor any of the other dyes produced any changes which exceeded significantly in magnitude the corresponding changes from normal as found in the tumor tissue.

TABLE 5

DISTRIBUTION OF PROTEIN-BOUND AMINOAZO DYE IN THE LIVER FRACTIONS OF RATS FED VARIOUS AMINOAZO DYES*†

(Azo Dye Fed)

FRACTION	4′-ME-DAB‡	DAB	3′-ME-DAB
	Micromoles×10^2 of dye per gram of fresh liver		
Whole homogenate	1.71–1.82	2.40–3.41	1.91–2.64
Nuclei	0.10–0.05	0.31–0.31	0.41–0.38
Large granules	0.22–0.16	0.38–0.49	0.18–0.14
Small granules	0.22–0.30	0.32–0.62	0.25–0.38
Supernatant fluid	1.01–1.45	1.67–1.88	1.09–1.53
Recovery	1.55–1.96	2.68–3.30	1.93–2.43
	Micromoles×10 of dye per gram of protein		
Whole homogenate	1.55–1.57	2.28–3.22	1.72–2.20
Nuclei	0.85–0.36	1.82–1.90	1.19–1.04
Large granules	0.58–0.48	1.42–1.67	1.08–0.70
Small granules	1.30–1.69	2.76–3.92	2.06–2.68
Supernatant fluid	2.40–3.20	3.76–4.57	2.64–3.14

* The first and second numbers in each column refer to the first and second series of experiments, respectively, as mentioned in the text.

† The non-specific absorption in the bound dye extracts was determined by carrying each of the protein samples from the liver fractions of the rats fed the basal diets through the bound dye determination. The absorption of these extracts at 520mμ gave the following corrections to be applied as log $I_0/I \times 10^3$ per 100 mgm. of protein: whole homogenate 57, nuclei 68, large granules 42, small granules 62, and supernatant fluid 35. The corrected values for log I_0/I were converted to micromoles of dye as described previously (3).

‡ DAB = 4-dimethylaminoazobenzene.

There does not appear to be any distinct relationship between the intracellular distribution of the protein-bound dye formed by any of the dyes studied here and their carcinogenic potencies. While it will be necessary to go far beyond these relatively gross morphological boundaries to identify the proteins involved, it is clear that initial cell fractionation will be of great value in any further study.

SUMMARY

1. The livers of rats fed no azo dye or equimolar levels of either 4-aminoazobenzene, 4′-methyl-4-dimethylaminoazobenzene, 4-dimethylaminoazobenzene, or 3′-methyl-4-dimethylaminoazobenzene for four weeks were homogenized and separated by differential centrifugation into nuclear, large granule, small granule, and supernatant fluid fractions. The original homogenate and the fractions were analyzed for protein, nucleic acids, riboflavin, and protein-bound aminoazo dye.

2. The non-carcinogenic dye, 4-aminoazobenzene, and the weakly carcinogenic dye, 4′-methyl-4-dimethylaminoazobenzene, produced little or no change in the composition of the liver.

3. The highly active carcinogen, 3′-methyl-4-dimethylaminoazobenzene, produced many of the same changes in the composition of the fractions as were observed previously with 4-dimethylaminoazobenzene, a moderately strong carcinogen, except that the changes were greater in magnitude. These changes included increased levels of protein and desoxypentosenucleic acid in the nuclear fraction, decreased contents of protein and pentosenucleic acid in the large and small granules, and a decreased amount of riboflavin in the large granules. In addition this powerful carcinogen increased the level of pentosenucleic acid in the nuclear fraction and the supernatant fluid. Many of the changes induced by feeding 3′-methyl-4-dimethylaminoazobenzene were so great as to make the liver very similar to hepatic tumor tissue in intracellular composition.

4. All of the dyes produced protein-bound aminoazo dye in each fraction of the liver cell. No correlation was evident between the intracellular distribution of bound dye and the carcinogenicity of the dye fed.

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Effect of Low Environmental Temperature, Dinitrophenol, or Sodium Fluoride on the Formation of Tumors in Mice*

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It has been demonstrated consistently that chronic caloric restriction inhibits the formation of various spontaneous and induced tumors of the mouse (1, 2). The mechanism of this effect is unknown. However some insight might derive if it were known whether the decreased caloric intake, the accompanying restriction in body weight, or the decreased total metabolism, was most directly related to the inhibition of tumor development.

The present investigation was undertaken to acquire information relevant to this question by examining the effects on tumor formation of experimental procedures which would result in retarded body growth, despite unchanged or augmented caloric intake. Two of the procedures were the feeding of dinitrophenol and housing of the mice at low environmental temperature. The third procedure was the feeding of sodium fluoride, inasmuch as under suitable conditions it effects a significant retardation in body weight without a parallel change in food consumption.

In these experiments four types of tumors were utilized—spontaneous mammary carcinoma, induced sarcoma, induced skin tumor, and primary lung adenoma. Only the spontaneous mammary carcinoma was investigated with all three experimental procedures; the results have been reported previously (3).

GENERAL METHODS

The following methods and conditions were common to all the experiments. The mice either were obtained from the Roscoe B. Jackson Memorial Laboratory (in which case the strain designation is preceded by JAX) or were bred in our laboratory by brother to sister mating. They were fed Purina fox chow or dog chow checkers from weaning (or when received in the laboratory) until the beginning of the experiment, generally when the mice were 7 to 12 weeks of age. Since the study was performed over a period of years, the foodstuffs employed in preparing the diets differed among the various series of experiments. In all instances the control diets supported relatively good growth and health. The rations were prepared by mixing the weighed foodstuffs with sufficient water to make easily molded mashes which were spread in pans and cut into blocks of appropriate size. When sodium fluoride (C.P.) or sodium salt of 2,4 dinitrophenol (Eastman-Kodak) was fed it was dissolved in water and incorporated into the mash. In the earlier experiments the diets were made daily; it was later found expedient to prepare them weekly, and store them in a refrigerator. The diets were fed daily; by weighing the food remaining in the cages at the end of each week, the actual food consumptions were estimated. Drinking water was available at all times.

The mice were housed in groups of five in cages with solid bottoms. Each animal was numbered and a separate record of its progress was kept. They were weighed and inspected for tumors at 2 week intervals except during the period in which the tumors appeared rapidly, when they were inspected weekly. The animals were examined postmortem—at sacrifice when the tumors became very large, at death, or at the termination of the experiment. The lesions were recognized as tumors by their appearance and progressive growth; the tumor type was established by gross examination and sectioning. Histological examinations were made of many tumors selected at random and of the few doubtful lesions. In general, the experiments were terminated either when the rate of formation of tumors had passed its peak and very few new tumors were appearing, or when the surviving animals were too few to modify the nature of the results.

EXPERIMENTS

Spontaneous Mammary Carcinoma

Experiment 1.—Each of the four groups was composed of 50 dba female mice born in the laboratory; litter-mates were distributed among the groups. The experimental diets consisted of 35 per cent Purina fox chow meal, 24 per cent skimmed milk powder, and 41 per cent cornstarch; they were instituted when the mice were 7 to 10 weeks old. The mice of the control group, P40, and of group P50 were fed the described ration; the diet fed group P47 contained sodium fluoride at the

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level of 0.09 per cent; and that fed group P48 contained 0.25 per cent of the sodium salt of 2,4 dinitrophenol. When the mice of group P50 were 19 to 22 weeks old they were transferred to a room kept between 45 and 55° F.; this contrasts with the average temperature of 80° F. in the laboratory in which the other three groups were housed.

The mice were fed 3.4 gm. daily and during the main course of the experiment the average daily food consumptions were: control group, 3.0 gm.; sodium fluoride group, 2.7 gm.; sodium dinitrophenol group, 3.3 gm.; and cold room group, 3.4 gm. At first the mice fed the ration containing dinitrophenol (P48) ate less food than the control mice and lost considerable weight. Within a few weeks, however, they increased their food consumption and returned to their original body weights (Fig. 1). The early weight loss may have been due to a distaste for the ration since a scout experiment indicated that the initial decrease in food consumption was proportional to the concentration of dinitrophenol in the ration. The control mice and those in the cold room drank about the same amount of water. The average water intake of the mice fed sodium fluoride was about 100 per cent greater, and that of those fed dinitrophenol about 30 per cent less, than that of the controls. The mice fed sodium fluoride or dinitrophenol, or housed at low environmental temperature, maintained body weights considerably lower than those of the control group (Fig. 1).

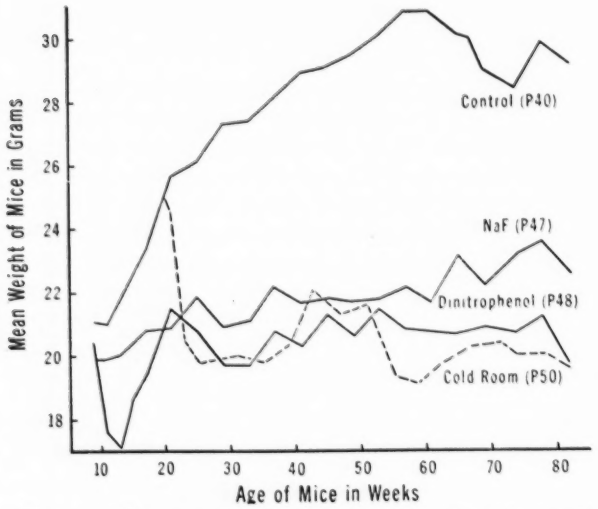

FIG. 1.—The growth of mice fed sodium fluoride (0.09% of diet) or dinitrophenol (0.25% of diet), or housed at low environmental temperature (45°–55° F.). Experimental conditions are given in text.

When the mice housed in the cold room (P50) were about 80 weeks of age, it was observed that a few were comatose when inspected in the morning; such mice survived only if removed to the general laboratory (80° F.). Because this phenomenon was increasing in severity, the whole group was transferred to the general laboratory when the mice were 85 weeks old. This change, late in the experiment, had no effect on the rate of tumor formation.

The experiment was terminated when the surviving mice were 97 to 100 weeks old. The results are given in Figure 2 and Table 1. All three experimental procedures significantly inhibited tumor formation. The incidences of mammary tumors were: control group (P40), 74 per cent; sodium fluoride group (P47), 42 per cent; dinitrophenol group (P48), 2 per cent; and cold room group (P50), 10 per cent. The decreased incidence of tumors in the experimental groups was not due to an increased death rate among these mice. Even at 75 weeks there were, in each group, only 4 to 9 mice dead without tumors, yet the tumor incidences were 23, 2, and 8 per cent for the experimental groups P47, P48, and P50 respectively, in comparison with 46 per cent for the control group.

In this long-term experiment there were certain observations not directly related to the main objectives of the study. In the group fed sodium fluoride, 9 mice had chronic nephritis, and 33 of the animals, at one time or another after 1 year of age, developed "overgrown" teeth. The first few mice with "overgrown" teeth died from starvation; the rest were maintained by the simple expedient of clipping their teeth whenever necessary. In the group fed dinitrophenol, and the group housed at a low environmental temperature, the principal pathological change at death was pneumonia; the next most common finding, associated only in a few cases with pneumonia, was edema (hydrothorax, ascites, or anasarca).

Per Cent of non-tumor mice dead						
P40		15	15	30	46	77
P47	3	13	23	30	53	80
P48			10	25	45	78
P 50	2	7	13	29	49	64

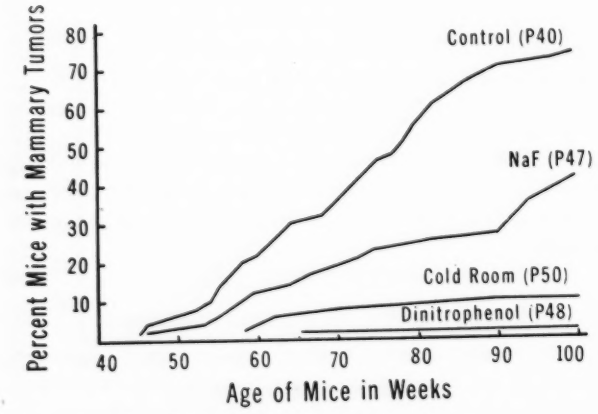

FIG. 2.—Cumulative incidences of spontaneous mammary carcinomas in strain dba mice fed sodium fluoride or dinitrophenol, or housed at low environmental temperature; based on "effective totals" of mice. The chart at the top of the figure gives the percentage of non-tumor mice dying before the ages indicated on the abscissa; *i.e.*, this indicates the death rate among only those mice that did not develop tumors.

INDUCED SARCOMA

In the following experiments sarcomas were induced by subcutaneous injection in the interscapular area of 3:4 benzpyrene dissolved in 0.2 cc. of an oily fraction of lard.

Experiment 2.—Three groups of 40 JAX-Swiss female mice, about 10 weeks old, were employed. Each mouse was injected with 0.15 mg. of benzpyrene. The control group (L30) was placed on a diet of cracked spring wheat, 64 per cent; Purina dog chow meal, 18 per cent; skimmed milk powder, 7 per cent; and white milled flour, 11 per cent. Group L7 was given the same diet but containing 0.25 per cent sodium salt of dinitrophenol. dinitrophenol mice about 30 per cent more water than did the control mice. The ingestion of the sodium fluoride or dinitrophenol rations resulted in decreased body weight (Table 2).

The experiment was terminated 52 weeks after the injection of carcinogen, more than 12 weeks after the last sarcoma had appeared in any group. There were no significant differences in tumor formation (Table 2).

TABLE 1

THE INHIBITING EFFECT OF SODIUM FLUORIDE, DINITROPHENOL, AND LOW ENVIRONMENTAL TEMPERATURE ON THE FORMATION OF SPONTANEOUS MAMMARY CARCINOMA IN STRAIN Dba MICE

GROUP*	NUMBER OF MICE		MICE DEVELOPING TUMORS			MEAN AGE AT TUMOR APPEARANCE (WEEKS)	NUMBER OF MICE§ TUMOR-FREE AND ALIVE AT END OF EXPERIMENT
	Effective†	Adjusted‡	Number	Per cent effective	Per cent adjusted		
P40: Control	50	47	37	74	79	71±2.3	3
P47: NaF	48	40	20	42	50	76±3.5	6
P48: DNP	50	42	1	2	2	‖	11
P50: Cold room	50	42	5	10	12	68±5.9	16

* NaF indicates 0.09% sodium fluoride in diet; DNP, 0.25% sodium 2,4-dinitrophenol in diet; cold room, mice housed at 45° to 55° F. between 22 and 85 weeks of age.

† Number of mice alive when first tumor was observed in experiment.

‡ Number of mice obtained by adjustment for the deaths of animals without tumors (4).

§ Experiment terminated when mice were 97 to 100 weeks old.

‖ The single tumor of this group appeared at 65 weeks.

TABLE 2

THE EFFECT OF DINITROPHENOL AND SODIUM FLUORIDE ON THE FORMATION OF BENZPYRENE-INDUCED SARCOMAS AND ON THE BODY WEIGHT OF THE MICE

EXPERIMENT*	GROUP†	NUMBER‡ OF MICE	MICE DEVELOPING SARCOMAS		TIME§ OF TUMOR APPEARANCE (WEEKS)		NUMBER OF MICE§ SARCOMA-FREE AND ALIVE AT END OF EXPERIMENT	MEAN BODY WEIGHTS‖ OF MICE (GM.)		
			Number	Per cent	Range	Mean		Weeks 0	16	40
2: JAX-Swiss female; 0.15 mg. benzpyrene	L30: Control	39	19	49	16–40	25±1.4	15	20	28	32
	L207: NaF	39	22	56	14–36	24±1.4	14	20	25	26
	L7: DNP	40	19	48	14–38	21±1.2	20	20	21	24
3: JAX-Swiss female; 1.5 mg. benzpyrene	L50: Control	40	20	60	12–26	18±0.9	14	20	28	32
	L407: NaF	39	25	64	14–42	21±1.4	11	20	23	22
4: JAX-ABC female; 0.1 mg. benzpyrene	L60: Control	40	7	18	15–43	28±3.5	29	18	32	39
	L67: NaF	40	13	33	17–51	31±2.8	25	19	24	21
5: C57 Black male; 0.15 mg. benzpyrene	A82: Control	49	30	61	13–39	24±1.2	18	25	37	40
	A88: DNP	50	30	60	15–39	25±1.2	18	25	23	24

* Experiment number, strain and sex of mice, and dosage of carcinogen administered subcutaneously.

† See footnote *, Table 1.

‡ Number of mice alive when first tumor was observed in experiment.

§ Experiments 2, 3, and 4 ended 52 weeks after injection of carcinogen; experiment 5 ended 40 weeks after injection.

‖ The mean weights are those of the mice without tumors; time in weeks after injection of benzpyrene.

Group L207 was fed the basic diet for 12 weeks, after which sodium fluoride was incorporated at a level of 0.09 per cent.

Each mouse was given 4.5 gm. of its experimental diet daily. The control mice ate an average of 3.3 gm. daily, those fed sodium fluoride about 3.6 gm., and those fed dinitrophenol all the food given them, 4.5 gm. The sodium fluoride mice drank twice as much, and the

Experiment 3.—Two groups of 40 JAX-Swiss female mice about 10 weeks of age were employed. Each mouse was injected with 1.5 mg. of benzpyrene. The mice of group L50 were fed the same basic diet employed in experiment 2; those of group L407 were fed the same diet for 12 weeks, when sodium fluoride was added at the level of 0.09 per cent. As in the preceding experiment the mice fed sodium fluoride ate slightly more food (on the

average 3.5 gm. daily compared to 3.2 gm.) yet their growth was retarded. They drank more than twice as much water as the control mice.

The experiment was terminated 52 weeks after the injection of the carcinogen, 10 weeks after the last sarcoma had appeared. Fifty per cent of the control mice developed tumors compared with 64 per cent in the sodium fluoride group (Table 2).

Experiment 4.—Two groups of 40 JAX-ABC female mice about 9 weeks old were utilized. One-tenth mg. benzpyrene in 0.2 cc. lard was injected subcutaneously. The diets were the same as those of experiment 3; feeding of sodium fluoride was begun 7 weeks after injection of the carcinogen. The mice of the group given sodium fluoride, L67, ate somewhat less than those of the control group, L60 (3.2 gm. compared with 3.5 gm. daily); they drank about twice as much water. Their body weights were considerably less than those of the controls.

The experiment was terminated 52 weeks after injection of the carcinogen inasmuch as few sarcomas had formed after the forty-third week (2 in the fluoride group, none in the control group). As in the preceding two experiments there were few non-tumor deaths. Thirty-three per cent of the mice in the fluoride group and 18 per cent in the control group developed sarcomas.

Experiment 5.—The effect of dinitrophenol on the formation of sarcomas was reexamined. Two groups of C57 Black male mice were employed; they were 9 to 18 weeks old at the beginning of the study when each mouse was injected with 0.15 mg. of benzpyrene. The ration consisted of Purina fox chow meal, 35 per cent; skimmed milk powder, 23 per cent; and cornstarch, 42 per cent; fed at a level of 4.0 gm. daily to the mice of group A82. The mice of group A88 were given the same diet, but containing 0.25 per cent sodium salt of dinitrophenol.

The dinitrophenol-treated mice ate about 20 per cent more food (average of 3.8 compared with 3.2 gm.) and drank about 30 per cent more water. The control mice grew to an average weight of 40 gm. while those fed dinitrophenol hardly maintained their initial weight of 25 gm. (Table 2). The experiment was terminated 40 weeks after the injection of carcinogen as the rate of appearance of tumors had definitely decreased. There was no difference in tumor formation between the two groups.

Summary of sarcoma experiments.—In experiment 1 the addition of sodium fluoride or dinitrophenol to the diet resulted in a striking reduction in the incidence of spontaneous mammary carcinomas. In contrast, diets containing the same levels of sodium fluoride or dinitrophenol had no inhibiting effect on the formation of benzpyrene-induced sarcomas; actually in the three experiments with sodium fluoride there is a consistent suggestion of a slight augmentation of sarcoma formation. There was no decrease in the incidence of sarcomas even though the body weights of the experimental mice ranged, on the average, from 10 to 40 per cent less than those of the control mice.

Induced Skin Tumors

Experiment 6.—Two groups of 45 JAX-Swiss female mice about 10 weeks old were used. The basic ration was of the same composition as that of experiments 2, 3, and 4. The mice of the control group, K2, were fed 4.5 gm. per day; group K28 was fed the same kind and amount of diet but containing sodium dinitrophenol at the level of 0.27 per cent. Four days after the diets were instituted the mice were given the first application of carcinogen, a single drop of a 0.3 per cent solution of benzpyrene in benzene, to the interscapular area; 32 such applications were given in 21 weeks. The mice fed dinitrophenol consumed nearly all of the 4.5 gm. of daily ration, while the mice of the control group ate an average of 3.3 gm. The dinitrophenol mice drank 30 per cent more water than the controls. Dinitrophenol retarded the growth of the mice despite increased food consumption (Table 3).

TABLE 3

THE EFFECT OF DINITROPHENOL ON THE FORMATION OF BENZPYRENE-INDUCED SKIN TUMORS AND ON THE BODY WEIGHT OF THE MICE

Group*	Number of mice†	Mice developing skin tumors		Time of tumor appearance (weeks)		Number of mice‡ tumor-free and alive at end of experiment	Mean Body Weights§ of mice (gm.) Weeks		
		Number	Per cent	Range	Mean		0	16	40
K2: Control	43	22	51	13–41	24±1.7	15	19	27	34
K28: DNP	42	18	43	13–39	24±1.6	21	20	22	25

* DNP indicates 0.27 per cent sodium salt of dinitrophenol in diet.

† Number of mice alive when first tumor was observed.

‡ Experiment terminated 42 weeks after first application of carcinogen.

§ The mean weights are those of mice without tumors; time in weeks after first application of carcinogen.

The experiment was terminated 42 weeks after the first application of carcinogen inasmuch as few skin tumors appeared after the thirty-fourth week. The data on formation of skin tumors are summarized in Table 3: the incidences were 51 per cent for the control mice, and 43 per cent for the mice in the dinitrophenol group.

Lung Adenoma

Experiments 2, 3, 4, and 6 were performed on strains of mice which spontaneously develop lung tumors (Swiss and ABC). At the termination of those experiments the autopsy observations were used to evaluate the effects of sodium fluoride or dinitrophenol on the incidence of grossly visible primary lung tumors; the few mice bearing other tumors were excluded. Either sodium fluoride or dinitrophenol, incorporated in the diet, caused a significant reduction in the incidence of

lung tumors (Table 4). These results were obtained in experiments in which there was no noteworthy inhibition in the incidence of either skin tumors or sarcomas.

The lung tumors have been designated as primary rather than spontaneous inasmuch as all the mice were treated with benzpyrene which may have augmented their rate of appearance (5, 6). The lung tumor incidence of group L50, experiment 3, was considerably higher than that of group L30, experiment 2, although the mice were of the same strain, sex, and age. This probably was due to the fact that group L50 was injected with ten times as much carcinogen.

DISCUSSION

The results of the experiments included in this publication indicate that, under the selected conditions, the feeding of sodium fluoride or of sodium 2,4 dinitrophenol, or exposing the mice to low environmental temperature significantly inhibits the formation of the spontaneous mammary carcinoma; the feeding of either of the two "growth-retarding chemicals" significantly inhibits the formation of primary lung adenoma; the two chemicals have little effect upon the formation of the induced sarcoma; and dinitrophenol has no significant effect upon the formation of the induced skin tumor.

TABLE 4

THE INHIBITING EFFECT OF DINITROPHENOL AND SODIUM FLUORIDE ON THE FORMATION OF PRIMARY LUNG TUMORS

Experiment	Group	Description	Age of mice (weeks)	Number of mice	Per cent with lung tumors
2 (JAX-Swiss)	L30	Control	62	15	33
	L7	Dinitrophenol		20	15
	L207	Sodium fluoride		14	7
3 (JAX-Swiss)	L50	Control	62	11	91
	L407	Sodium fluoride		10	40
4 (JAX-ABC)	L60	Control	60	29	34
	L67	Sodium fluoride		25	16
6 (JAX-Swiss)	K2	Control	52	15	53
	K28	Dinitrophenol		21	5

Low environmental temperature.—It is well known that warm-blooded animals tend to increase their food intake when living in the cold. For example, Schwabe, Emery, and Griffith (7) reported that rats housed at 45°–55° F. consumed approximately 30 per cent more food than the controls, yet they experienced some growth retardation. Donhoffer and Vonotzky (8) have shown that the caloric intake of mice is 10 to 20 per cent greater at 50° F., and 10 to 20 per cent lower at 90° F., than that of mice at room temperature. In our experiment, the mice housed at 45°–55° F. were given only 3.4 gm. of food daily (on the average about 10 per cent more than that eaten by the mice in 80° F. environment); they would have eaten more if food had been available. Possibly as a partial consequence of this relative restriction of caloric intake, the mice housed in the cold room maintained average body weights of 20 to 22 gm. compared with attained values of 29 to 30 gm. for the control mice.

Mills and co-workers have published results on the effects of the environmental temperature on the formation of tumors in mice (9, 10, 11). Although the factors of the individual experiments differed slightly, in general, groups of mice were housed at 68°, 79°, and 91° F. In early publications, no data on food consumption or body weight were presented, although it was stated that the mice at 91° F. were smaller; later data (12) suggest that both food consumption and body growth were decreased at 91° F. Spontaneous mammary tumors, in both dba and C3H strains, appeared earlier and in greater numbers in mice housed at 68° F. than in mice in the 91° F. room. In our experiment, an 80° F. environment was compared with one of 45°–55° F.; the mice in the latter environment developed considerably fewer tumors. The common point of agreement between the studies of Mills and associates and our own is that inhibition of the formation of spontaneous mammary carcinomas, in mice housed at low or high environmental temperatures, was associated with retarded body growth. Considering both studies, the effect on tumor formation does not appear to be directly related to the total food consumption.

Dinitrophenol.—Administration of dinitrophenol increases the metabolic rate mainly by direct action on the tissues. When fed continually at suitably high levels, it also induces increased food consumption and a loss of body weight (13). Our results are in agreement with these observations in that ingestion of 0.25 per cent sodium 2,4 dinitrophenol (8 to 10 mg. daily) in the ration produced a severe restriction in body growth despite increased food consumption. There was a considerable reduction in the incidences of spontaneous mammary carcinomas and primary lung adenomas but little or no effect upon the rates of appearance or incidences of induced sarcomas or skin tumors. On the other hand, Kreyberg (14) reported that skin tumors induced by tarring appeared at an accelerated rate in mice fed dinitro-*o*-cresol at levels from 0.1 to 2.0 mg. per day.

Sodium fluoride.—It has been shown (15) that sodium fluoride, fed to rats at levels of 0.1 per cent of the ration, effects a significant retardation in body weight without any accordant change in food consumption. In the sodium fluoride experiments described in the present paper, 0.09 per cent of the salt was incorporated into the diet. The average weights of the several experimental groups were about 10 to 40 per cent less than the weights of the corresponding control groups while the caloric intakes were within 10 per cent, greater or less, of the controls. Sodium fluoride considerably inhibited the incidence of spontaneous mammary carcinomas and of lung adenomas, but not of induced sarcomas. In fact, in the experiments with sarcomas, the fluoride-treated groups of mice had a slightly greater incidence of sarcomas than the mice of the corresponding control groups. The differences in the individual experiments were not of statistically significant magnitude but were consistently in the same direction.

In all of the experiments the mice ingesting 0.09 per cent sodium fluoride drank at least twice as much water as the control mice.

Implications of results.—Two main questions arise from this work: 1) Why did sodium fluoride or dinitrophenol affect the formation of spontaneous mammary carcinomas and lung adenomas differently from that of induced sarcomas and skin tumors? 2) Do the results of the present investigation give any insight into the mechanism by which restricted caloric intake inhibits the formation of tumors?

Feeding of sodium fluoride or dinitrophenol resulted in drastic retardation of body growth. Although, in each experiment, the average weight of the treated mice was considerably less than that of the control mice, the incidence of spontaneous mammary carcinomas and of lung adenomas was markedly reduced, while the formation of sarcomas and of skin tumors, induced by carcinogenic hydrocarbons, was not significantly affected. The discrepant effects of the feeding of sodium fluoride or dinitrophenol on tumor formation are further emphasized by the comparison between their slight influence on the incidences of sarcomas and skin tumors (in experiments 2, 3, 4, and 6) and the considerable reduction in the incidence of primary lung adenomas in the *mice of the same groups.* There are other instances in which an experimental procedure produced dissimilar effects on tumor formation depending on the neoplasm investigated. This is not unexpected since tumors are different diseases arising in different tissues under different conditions. For example, although caloric restriction inhibits the formation of all tumors tested to the present time, the spontaneously occurring mammary carcinoma, lung adenoma, and hepatoma are affected to a greater degree than are the induced epithelioma or sarcoma of the mouse (2, 16). Fat-enriched diets augment the formation of spontaneous mammary carcinoma and skin tumors induced by carcinogenic hydrocarbons, but have no effect on the incidence of spontaneous lung adenoma or of the sarcoma induced by carcinogenic hydrocarbons (17). Varying the proportion of dietary casein has a pronounced effect on the incidence of spontaneous hepatomas in mice but produces negligible change, if any, in the formation of spontaneous mammary tumors and induced skin tumors (18) or induced sarcomas (18, 19).

There appears to be no simple explanation for the diverse response of different tumor types to the experimental procedures employed in the present study. Possibly the most important determinants are the nature (exogenous or endogenous), potency, and period of action of the carcinogen. It is likely that the spontaneous mammary carcinoma and lung adenoma are produced by mild carcinogenic factors acting over a long period of time, as suggested by their relatively long latent periods. On the other hand, the induced skin tumor and sarcoma, here studied, are produced by relatively large doses of agents exerting their carcinogenic action over a shorter period; under these conditions, the experimental procedures probably are less able to modify the formation of tumors. There is evidence from other studies (20, 21, 22) suggesting that the use of smaller doses of carcinogenic hydrocarbons would increase the sensitivity of the response to the experimental procedures employed. Other factors, also suspect as causes of the diverse

response of various tumors, are the differential actions that both carcinogens and experimental procedures may have on various tissues.

It may be that under other experimental conditions, where low doses of sodium fluoride or dinitrophenol, or housing at slightly below "normal" temperature are employed, mice may consume somewhat more food than the controls and experience no significant restriction in body growth. Under such conditions there may even be some enhancement of tumor production, as suggested by Kreyberg's experience with dinitrocresol (14).

The central purpose of these investigations was not to disclose the effects of sodium fluoride, dinitrophenol, or low environmental temperature on tumor formation; rather, these growth-retarding procedures were utilized to study the mechanism by which caloric restriction inhibits tumor formation. Such investigations could not reveal the finer mechanism of the caloric effect, but might indicate whether decreased caloric intake, accompanying restriction in body weight, or decreased total metabolism is the factor most directly involved.

The results suggest that the amount of food consumed (caloric intake) itself is not the means through which caloric restriction inhibits tumor formation. In the present study the incidences of spontaneous mammary carcinomas and primary lung tumors were strikingly decreased, regardless of whether the food consumption values were greater or less than the corresponding values for control groups; the incidences of induced sarcomas and skin tumors also were not associated with food consumption values.

When the procedures resulted in decreased tumor incidence—in the experiments with the spontaneous mammary carcinoma and primary lung adenoma—the common feature was restricted growth of the animals. In the caloric restriction experiments, as well as those reported here, food intake was inadequate to meet the metabolic requirements of the animal. Neither the rate nor amount of metabolic turnover appears to be the primary factor in the incidence of tumors. What seems to be of significance is the body weight at which a balance is struck between caloric intake and expenditure. If this is struck at a high level of body weight the incidence of tumors is high; if at a low level of body weight the incidence is low. Other dietary modifications, such as limiting the proportion of protein or amounts of riboflavin to levels inadequate for body growth, also result in decreased incidence of these tumors (23, 24, 25, 26). Thus, so far as caloric restriction is concerned, *the inhibitory effect on tumor formation may be associated with the limited body weight.* On the other hand, the absence in the present study of inhibition of formation of sarcomas or skin tumors, despite considerable reduction in body weight, limits such an interpretation unless recourse is had to explanations special to the experimental procedures or tumors.

SUMMARY

The present study was undertaken to determine whether chronic caloric restriction inhibits the formation of tumors through the decreased caloric intake, the associated retardation of body growth, or the reduced total metabolism. Procedures that resulted in retarded body growth despite unchanged or augmented food consumption and total metabolism were investigated: housing mice at low environmental temperature, or feeding them either dinitrophenol or sodium fluoride. Four tumors of the mouse were utilized, but only the mammary carcinoma was investigated with all three experimental procedures.

Under the selected conditions, housing the mice at 45°–55° F., or feeding them either 0.25 per cent sodium 2,4 dinitrophenol or 0.09 per cent sodium fluoride, significantly inhibited the formation of spontaneous mammary carcinomas; the feeding of either of the two chemicals significantly inhibited the formation of primary lung adenomas; the two chemicals had little effect upon the formation of induced sarcomas; and dinitrophenol did not appreciably alter the incidence of induced skin tumors.

The diverse response of the mammary and lung tumors in comparison with the sarcoma and skin tumor is discussed in relation to experiences with procedures other than those employed in this study. The present data suggest that neither the food consumption (caloric intake) nor the amount or rate of metabolic turnover are consistently related to tumor formation. However, the results with the mammary carcinoma and lung adenoma imply that the inhibition of tumor formation brought about by caloric restriction is associated with the low weight of the animals.

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Cancer Mortality Among Males and Females in Denmark, England, and Switzerland

IV. Mortality of Accessible and Inaccessible Cancers in Danish Towns and Rural Areas

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Previous studies, recorded in detail (1, 2), showed that in the years immediately before World War II there was a clear difference in sex distribution of cancer deaths between the three European countries of highest cancer mortality: Switzerland, England, and Denmark.

For a further analysis the three European materials were subdivided into cancers of accessible and inaccessible site (3), the latter taken both as comprising and as excluding gastric cancer. This subdivision was made because we expected that international differences in diagnostic methods and clinical tradition would have most influence on the figures for cancers of inaccessible sites, which are equally inaccessible to diagnosis and treatment. Differences in deaths from cancers of accessible sites would probably be ascribable either to real differences in cancer incidence or to differences in therapeutical results, as the diagnosis of these cancers is less complicated.

Thus it was demonstrated, that exclusion of gastric cancer from inaccessible cancers in women eliminates the differences between the three countries concerned, whereas accessible female cancers show some differences in mortality which reasonably may be ascribed to differences in incidence, or perhaps in therapeutical results.

A corresponding exclusion of gastric cancer from the male inaccessible cancers did not eliminate inter-European differences, but if gastric cancer was eliminated from the total cancer mortality, both sexes showed that the differences between the two countries of highest cancer mortality (*viz.* for males Switzerland and England, and for females Denmark and England) disappeared. As these differences thus are ascribable to a cancer difficult to diagnose they may be taken with some reservation.

Danish death certificates from the same years, 1935 to 1939, showed similar differences in sex distribution according to age, if subdivided into Capital, Provincial Towns, and Rural Areas, and it may now be of interest to compare the results of division into accessible and inaccessible sites with and without gastric cancer, with those from the inter-European comparison.

To test the validity of the differences demonstrated we have carried out a corresponding comparison for the death certificates from the years 1942 to 1944 with similar result, and thereafter we have repeated this on the material of the Danish Cancer Registry for the same period.

The methods employed follow in every detail those of the earlier reports, and consequently tables will be directly comparable with those of previous papers.

DEATH CERTIFICATES 1935 TO 1939

In dealing with Danish death certificates it should be mentioned that they are very uniform in quality. Public Health Insurance covers about 85 per cent of the population, and as nearly all hospitals are run by the public, the access to first-class medical attention is equal to all social strata. The percentage of death certificates issued by hospitals (2) has previously been given separately for Capital, Provincial Towns, and Rural Areas. It will be seen that there is some difference in the percentage of hospitalization, especially between Capital and Rural Areas, and the slightly higher quality of the Capital material is also reflected in the shape of the curves for the oldest age classes. Thus it will be seen that the curve for cancer deaths in the Capital (2, Fig. 5) continues its rise through the oldest age classes, while the curves for Rural Areas tend to fall in these classes, presumably as an expression of less hospitalization, and consequently less energetic diagnosis in the Rural Areas for the age classes concerned, which, however, are only of slight numerical importance.

Females.—It should be kept in mind, that differences in female cancer mortality between Danish Capital, Provincial Towns, and Rural Areas, although statistically significant, are less pronounced than those in male cancer mortality (2). However, female cancer of inaccessible site, whether or not including gastric cancer (Tables 2 and 2A and 3 and 3A), does not show any significant difference between the Danish country parts in question, so that—contrary to the inter-European comparison—we cannot ascribe the differences found to any difference in the occurrence of the diagnosis of gastric cancer.

With regard to accessible female cancer there is, however, a difference to be found (Table 4) as was the case in the European material, the Capital showing a higher mortality than Rural Areas. It is worth while noticing that the age classes concerned are ten years younger than the corresponding classes for male accessible cancers (Tables 4 and 4A and 8 and 8A), presumably because the female figures are an expression of mortality mainly from uterine and mammary cancer, which usually occur at an earlier age than cancers of the rectum and the oesophagus which will be prevalent as male cancers of accessible sites.

Especially with regard to uterine and breast cancer, however, hospital facilities are very uniform throughout Denmark, and to the authors it seems more than probable that differences in mortality from these sites of cancer are mainly due to a difference in incidence, and not to any difference in therapeutic or diagnostic results. This is in good correspondence with our more tentative suggestion to the same effect with regard to the differences in mortality from accessible female cancers, between the three European countries examined.

Males.—With regard to the male cancer mortality in Denmark, it seems that neither subdivision into cancers of accessible and inaccessible sites (Tables 5, 6, 7 and 8 and 5A, 6A, 7A and 8A) nor the exclusion of gastric cancer will influence the excess mortality in towns to any degree of statistical significance.

SUMMARY AND CONCLUSION

Previous studies on cancer mortality in Danish Capital, Towns, and Rural Areas have been continued, and the material of death certificates subdivided into cancers of accessible and inaccessible sites, the latter category comprising and excluding gastric cancer. These comparisons have been carried out on material from the period 1935 to 1939 from which period comparisons between Danish, English, and Swiss materials were carried out in previous papers.

Female cancers show a higher mortality from cancer of accessible sites in Capital than in Rural Areas.

Female cancers of inaccessible sites show the same incidence in all three country parts.

Male cancers of all subdivisions show a higher mortality in the Capital than in Rural Areas.

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DENMARK: 1935 TO 1939. CANCER MORTALITY PER 10,000 LIVING
(Cancer of All Sites, Except Stomach)

TABLE 1

FEMALES

Age	Capital	Provincial Towns	Rural Areas
0–19	0.2	0.2	0.3
20–24	0.4	0.4	0.4
25–29	1.0	1.3	1.1
30–34	2.5	2.8	2.3
35–39	5.8	5.8	4.9
40–44	11.8	9.5	7.3
45–49	19.1	15.8	13.3
50–54	25.8	20.0	19.1
55–59	36.6	30.0	28.0
60–64	44.3	39.4	33.3
65–69	51.6	52.0	46.5
70–74	68.6	70.5	63.6
75–79	88.1	83.6	83.4
80–84	105.5	91.5	96.6
85–	133.3	110.8	91.7

TABLE 5

MALES

Capital	Provincial Towns	Rural Areas
0.2	0.3	0.2
0.4	0.7	0.6
0.7	0.4	0.7
1.1	1.3	1.3
2.4	2.0	1.7
5.4	3.0	3.1
7.6	7.1	5.1
18.1	11.3	7.9
30.6	15.6	14.7
43.7	31.2	24.4
63.4	48.5	37.0
89.0	66.3	61.5
117.3	85.6	86.9
109.5	97.0	96.7
141.5	87.6	89.7

DENMARK: 1935 TO 1939. CANCER MORTALITY PER 10,000 LIVING
(Cancer of Inaccessible Sites, Including Stomach)

TABLE 2

FEMALES

Age	Capital	Provincial Towns	Rural Areas
0–19	0.2	0.2	0.3
20–24	0.3	0.4	0.2
25–29	0.6	0.8	0.5
30–34	1.0	0.9	1.0
35–39	2.3	1.9	2.4
40–44	5.0	4.1	4.4
45–49	8.9	7.8	6.9
50–54	12.4	12.0	12.1
55–59	20.0	18.7	20.4
60–64	29.7	34.7	28.4
65–69	42.5	49.2	49.0
70–74	67.5	79.0	73.9
75–79	93.3	103.2	105.4
80–84	111.6	106.0	128.7
85–	128.2	116.9	96.9

TABLE 6

MALES

Capital	Provincial Towns	Rural Areas
0.2	0.3	0.2
0.4	0.5	0.5
0.2	0.1	0.6
1.0	1.0	0.8
2.4	2.3	1.6
6.1	3.4	3.8
8.3	7.6	6.8
18.6	12.3	11.6
26.7	20.8	20.3
43.8	40.6	33.6
63.4	64.3	55.1
96.5	92.1	85.5
135.0	116.5	121.0
133.7	115.2	123.0
163.1	100.8	107.5

DENMARK: 1935 TO 1939. CANCER MORTALITY PER 10,000 LIVING
(Cancer of Inaccessible Sites, Stomach Excluded)

TABLE 3

FEMALES

Age	Capital	Provincial Towns	Rural Areas
0–19	0.2	0.2	0.3
20–24	0.3	0.4	0.2
25–29	0.6	0.7	0.4
30–34	0.8	0.7	0.7
35–39	1.8	1.5	1.8
40–44	4.0	3.0	2.9
45–49	7.3	5.7	4.6
50–54	9.9	8.5	8.5
55–59	15.1	12.2	13.5
60–64	20.0	21.1	16.5
65–69	27.1	25.5	24.9
70–74	38.4	41.2	33.9
75–79	53.9	51.0	46.9
80–84	64.8	48.3	54.4
85–	82.1	54.2	47.4

TABLE 7

MALES

Capital	Provincial Towns	Rural Areas
0.2	0.3	0.2
0.3	0.5	0.5
0.2	0.1	0.5
0.7	0.8	0.7
1.6	1.6	1.1
4.7	2.0	2.0
6.8	4.9	3.5
12.5	7.8	5.3
18.4	10.6	9.4
27.8	20.4	16.2
37.6	32.2	24.9
56.9	43.0	41.9
80.4	52.9	56.4
75.4	58.6	58.4
101.5	44.6	52.3

DENMARK: 1935 TO 1939. CANCER MORTALITY PER 10,000 LIVING
(Cancer of Accessible Sites)

TABLE 4

FEMALES

Age	Capital	Provincial Towns	Rural Areas
0–19	0.2		0.2
20–24	0.5	0.7	0.7
25–29	1.7	2.2	1.6
30–34	4.0	4.2	3.1
35–39	7.8	6.6	4.4
40–44	11.8	10.1	8.8
45–49	16.0	11.5	10.6
50–54	21.5	17.8	14.5
55–59	24.3	18.3	16.8
60–64	24.4	26.5	21.6
65–69	30.1	29.3	29.7
70–74	34.2	32.6	36.5
75–79	40.7	43.3	42.1
80–84	51.3	56.6	44.3
85–			

TABLE 8

MALES

Capital	Provincial Towns	Rural Areas
0.1	0.2	0.1
0.5	0.3	0.3
0.4	0.5	0.6
0.7	0.3	0.6
0.7	1.1	1.1
1.3	2.2	1.5
5.6	3.5	2.7
12.2	5.1	5.3
15.9	10.8	8.2
25.8	16.3	12.1
32.1	23.3	19.6
36.9	32.7	30.5
34.2	38.4	38.4
40.0	43.0	37.4

TABLE 1A

TEST OF SIGNIFICANCE: FEMALES
(Cancer of All Sites, Except Stomach)*

AGE	cap.-pr.t.	pr.t.-r.a.	cap.-r.a.†
0–19	−0.04	−1.88	−1.75
20–24	0.34	−0.30	0.08
25–29	−0.98	0.78	−0.21
30–34	−0.67	1.12	0.40
35–39	−0.72	1.36	1.28
40–44	1.96⊕	2.52⊕	5.00⊕⊕⊕
45–49	2.10⊕	1.95	4.52⊕⊕⊕
50–54	3.10⊕⊕	0.60	4.31⊕⊕⊕
55–59	2.73⊕⊕	0.98	4.26⊕⊕⊕
60–64	1.63	2.49⊕	4.51⊕⊕⊕
65–69	−0.12	1.74	1.64
70–74	−0.39	1.59	1.14
75–79	0.66	0.03	0.80
80–84	1.40	−0.58	1.01
85–	1.34	1.47	2.98⊕⊕

* All A tables are given in values of u. The statistical differences found in the tables giving the values of u are designated as follows:

1. no sign: $u < 1.96$, 5 per cent $< P$
2. ⊕ : $1.96 < u < 2.58$, 1 per cent $< P < 5$ per cent
3. ⊕⊕ : $2.58 < u < 3.29$, 1 per thousand $P < 1$ per cent
4. ⊕⊕⊕ : $3.29 < u$ $P < 1$ per thousand

where P is the probability of finding the observed or more widely differing values under the assumption that the true rates were the same in the groups compared.

† Designations in A tables here and in paper V are cap. = Capital; pr. t. = Provincial Towns; r. a. = Rural Areas.

TABLE 2A

TEST OF SIGNIFICANCE: FEMALES
(Cancer of Inaccessible Sites, Including Stomach)

cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
0.14	−1.88	−1.56
−0.38	1.00	0.58
−0.55	1.16	0.59
0.09	−0.10	−0.01
0.84	−1.07	−0.16
1.29	−0.60	0.88
1.05	1.00	2.28⊕
0.28	−0.08	2.46⊕
0.72	−1.08	−0.29
−1.92	2.79⊕⊕	0.63
−1.91	0.06	−2.13⊕
−2.26⊕	1.12	−1.46
−1.36	−0.32	−1.89
0.53	−2.36⊕	−1.76
0.68	1.46	2.20⊕

TABLE 3A

TEST OF SIGNIFICANCE: FEMALES
(Cancer of Inaccessible Sites, Stomach Excluded)

AGE	cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
0–19	0.14	−1.82	−1.50
20–24	−0.66	1.00	0.28
25–29	−0.35	1.34	1.00
30–34	0.42	−0.26	0.20
35–39	0.57	−0.70	−0.07
40–44	1.58	0.21	2.29⊕
45–49	1.78	1.46	3.62⊕⊕⊕
50–54	1.17	0.04	1.42
55–59	1.88	−0.96	1.20
60–64	−0.52	2.62⊕⊕	2.10⊕
65–69	0.62	0.29	1.04
70–74	−0.74	2.24⊕	1.44
75–79	0.53	1.00	1.56
80–84	2.20⊕	−0.98	1.52
85–	2.25⊕	0.74	3.32⊕⊕⊕

TABLE 4A

TEST OF SIGNIFICANCE: FEMALES
(Cancer of Accessible Sites)

cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
−1.26	−0.44	−1.54
2.37⊕	−2.66⊕⊕	−0.20
−0.93	−0.14	−1.18
−1.05	1.46	0.36
−0.39	2.10⊕	1.70
1.36	2.94⊕⊕	4.72⊕⊕⊕
1.31	1.34	2.64⊕⊕
3.12⊕⊕	0.79	4.48⊕⊕⊕
2.00⊕	2.18⊕	4.68⊕⊕⊕
2.74⊕⊕	0.88	4.18⊕⊕⊕
−0.80	2.19⊕	1.32
0.26	−0.12	0.17
0.40	−1.08	−0.64
−0.40	0.20	−0.25
−0.48	1.34	0.76

TABLE 5A

TEST OF SIGNIFICANCE: MALES
(Cancer of All Sites, Except Stomach)

AGE	cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
0–19	−1.04	0.56	−0.72
20–24	−1.06	0.24	−1.01
25–29	1.50	−1.68	0.00
30–34	−0.62	0.20	−0.53
35–39	0.80	0.62	1.59
40–44	3.06⊕⊕	−0.14	3.62⊕⊕⊕
45–49	0.50	2.41⊕	3.03⊕⊕
50–54	4.17⊕⊕⊕	3.05⊕⊕	8.02⊕⊕⊕
55–59	6.66⊕⊕⊕	0.62	9.02⊕⊕⊕
60–64	4.04⊕⊕⊕	3.08⊕⊕	8.02⊕⊕⊕
65–69	3.36⊕⊕⊕	3.74⊕⊕⊕	7.72⊕⊕⊕
70–74	3.62⊕⊕⊕	1.04	5.32⊕⊕⊕
75–79	3.50⊕⊕⊕	−1.98⊕	3.95⊕⊕⊕
80–84	0.96	0.03	1.12
85–	2.36⊕	−0.15	2.66⊕⊕

TABLE 6A

TEST OF SIGNIFICANCE: MALES
(Cancer of Inaccessible Sites, Including Stomach)

cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
−0.90	0.43	−0.66
−0.65	−1.12	−0.88
1.02	−3.08⊕⊕	−2.15⊕
0.01	0.60	0.64
0.06	1.84	1.98⊕
3.29⊕⊕⊕	−0.60	3.40⊕⊕⊕
0.73	0.94	1.70
3.75⊕⊕⊕	0.62	5.14⊕⊕⊕
2.66⊕⊕	0.29	3.48⊕⊕⊕
0.98	2.76⊕⊕	3.96⊕⊕⊕
−0.20	2.50⊕	2.17⊕
0.63	1.24	1.91
1.83	−0.58	1.62
1.28	−0.74	0.84
2.68⊕⊕	−0.44	2.56⊕

TABLE 7A

TEST OF SIGNIFICANCE: MALES
(Cancer of Inaccessible Sites, Stomach Excluded)

AGE	cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
0–19	−0.82	0.43	−0.66
20–24	−0.92	0.00	−1.08
25–29	1.58	−2.92⊕⊕	−1.38
30–34	−0.32	0.62	0.28
35–39	0.00	1.56	1.58
40–44	4.02⊕⊕⊕	−0.03	4.99⊕⊕⊕
45–49	2.00⊕	1.98⊕	4.46⊕⊕⊕
50–54	3.52⊕⊕⊕	2.75⊕⊕	7.12⊕⊕⊕
55–59	4.55⊕⊕⊕	0.99	6.60⊕⊕⊕
60–64	2.98⊕⊕	2.38⊕	5.94⊕⊕⊕
65–69	1.52	2.92⊕⊕	4.68⊕⊕⊕
70–74	2.76⊕⊕	0.28	3.57⊕⊕⊕
75–79	3.74⊕⊕⊕	−0.67	3.83⊕⊕⊕
80–84	1.60	0.03	1.88
85–	3.19⊕⊕	−0.74	3.14⊕⊕

TABLE 8A

TEST OF SIGNIFICANCE: MALES
(Cancer of Accessible Sites)

cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
−1.10	0.02	−0.74
−0.50	0.50	−1.16
0.82	0.42	1.44
−0.60	−0.40	−1.08
1.64	−1.38	0.51
−1.10	−0.19	−1.48
−1.62	1.40	−0.52
2.33⊕	1.34	4.18⊕⊕⊕
5.36⊕⊕⊕	−0.29	6.31⊕⊕⊕
2.77⊕⊕	2.01⊕	5.82⊕⊕⊕
3.53⊕⊕⊕	2.35⊕	6.61⊕⊕⊕
2.34⊕	1.41	4.08⊕⊕⊕
0.80	0.56	1.48
−0.54	0.01	−0.60
−0.21	0.60	0.22

Cancer Mortality Among Males and Females in Denmark, England, and Switzerland

V. Incidence of Accessible and Inaccessible Cancers in Danish Towns and Rural Areas

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DEATH CERTIFICATES 1942 TO 1944

Results given in previous papers I to IV have all been obtained by examination of the material of Danish death certificates from the years 1935 to 1939, which period alone would allow comparisons with England and Switzerland. It would now seem worth while to examine whether these results will hold good on a registration material which, better than death certificates, represents the real incidence of cancer. As, however, the Danish Cancer Registry was not started before 1942, we had to make sure that the results obtained on the material from 1935 to 1939, would be found also on a material of death certificates for 1942 to 1944.

That this is the case was evident from a comparison carried out with the technic of the preceding article.

MATERIAL OF THE DANISH CANCER REGISTRY

The system for the collection of the material has been described elsewhere in detail (4). Suffice it to say, that all cases of malignant disease admitted in Danish hospitals are registered with the Cancer Registry. The few cases from private practice will become known through death certificates, and the influence of this dualism in the material has previously been estimated and reported upon (5).

From the Tables 1A to 10A it will appear that the results obtained by means of death certificates are in the main borne out by the investigation based on the registration material, which gives the true incidence of cancer in a country as far as is possible with today's medical facilities.

Females.—Female cancers of accessible sites (Table 5A) show higher incidence in Capital than in Rural Areas, and the difference is rather more pronounced in the present material than shown by death certificates (IV, Table 4A). This seems to make further analysis worth while. However, "inaccessible sites" show no differences even after exclusion of gastric cancer (Tables 3A and 4A), which in spite of its own slight difference in incidence between Capital and Rural Areas does not influence inaccessible cancers as a whole. Similarly the exclusion of gastric cancer does not influence the differences within the group "cancers of all sites" (Tables 1A and 2A).

Males.—On the whole the incidence of accessible cancers among males is somewhat higher in Capital than in Rural Areas, but this difference seems less pronounced in the registration material (Table 10A). Contrary to the female material "inaccessible sites" shows a higher incidence for the Capital, uninfluenced by the subtraction of the figures for gastric cancer (Tables 8A and 9A), which seem remarkably uniform in the three country parts (Tables 11A and 12A), so that logicaly the elimination of gastric cancer leaves the figures for all sites uninfluenced and with a pronounced excess in the Capital.

Special sites.—If the numerically important sites of cancer are subject to further analysis we may have a hope of ascribing some of the differences found to certain sites of cancer. From Tables 13A to 16A it will appear, that the variation in the incidence of female cancers of accessible sites, even if shared by breast cancer and cancer of the uterine body, is in the main caused by a difference in the incidence of cervical cancer. It may here be added that 68.3 per cent of uterine cancers are treated in the three Radium Hospitals which in Denmark accept cases from Towns and Rural Areas as well. Furthermore, the percentage of cases known from death certificates only is so low (5) that it must be considered as certain that there is a real difference in the incidence of cervical cancer between Capital and Rural Areas.

For male cancers it seems most surprising that skin cancer does not seem to contribute to the ex-

cess of cancer in towns (Table 18A). For the time being we should think it inadvisable to enter into a further analysis of the male cancers of other sites, less frequently represented in the material.

SUMMARY AND CONCLUSION

Results of studies on cancer mortality described in the previous paper have been confirmed on a corresponding material from 1942 to 1944, and finally tested on a registration material from these years collected by the Danish Cancer Registry.

Female cancers show a higher incidence in accessible sites in the Capital than in Rural Areas, and this is mainly ascribable to a variation in incidence of cervical cancer, although mammary cancer and body cancer of the uterus display the same features, but less pronounced.

Female cancers of inaccessible sites show the same incidence in all the three country parts.

Male cancers of all subdivisions show a higher incidence in the Capital than in Rural Areas. Among the cancers of special site both gastric cancer and skin cancer show the same incidence in all country parts.

Thus we see that some of the inter-European differences found in the mortality material from 1935 to 1939 can be demonstrated within the borders of a small country, where it moreover has been possible to confirm them by examination of death certificates from 1942 to 1944, and comparison with a registration material from these years.

It seems defensible to conclude that a large part of the inter-European differences are ascribable to gastric cancer and thus probably mainly due to differences in diagnostic procedure and traditions for nomenclature, *etc.*

Likewise it seems probable that differences in the mortality from female accessible cancers represent real differences in incidence mainly ascribable to the differences in the occurrence of cervical cancer.[1]

[1] For references, see preceding paper IV.

DANISH CANCER REGISTRY: 1942 TO 1944
CANCER MORBIDITY PER 10,000 LIVING
(Cancer of All Sites, Including Stomach)

TABLE 1 — FEMALES

Age	Capital	Provincial Towns	Rural Areas
25–29	2.8	3.3	2.9
30–34	6.6	7.8	6.8
35–39	13.6	13.6	10.1
40–44	25.4	21.1	16.1
45–49	35.2	33.8	26.6
50–54	38.8	38.6	32.8
55–59	59.2	58.1	45.8
60–64	68.8	67.1	61.9
65–69	91.4	89.3	80.3
70–74	127.0	116.1	120.5
75–79	143.6	155.6	149.4
80–84	174.1	186.8	174.6
85–	218.9	178.1	188.0

TABLE 6 — MALES

Capital	Provincial Towns	Rural Areas
2.1	2.2	1.4
2.8	3.0	3.2
5.8	6.8	4.0
10.0	9.5	7.2
17.4	15.6	13.1
29.2	24.2	24.0
53.2	36.4	33.5
75.3	62.0	54.4
106.2	93.9	82.1
154.6	134.3	117.9
200.0	169.7	149.6
239.1	216.2	179.1
265.1	164.4	188.7

DANISH CANCER REGISTRY: 1942 TO 1944
CANCER MORBIDITY PER 10,000 LIVING
(Cancer of All Sites, Stomach Excluded)

TABLE 2 — FEMALES

Age	Capital	Provincial Towns	Rural Areas
25–29	2.8	3.1	2.9
30–34	6.1	7.5	6.6
35–39	13.3	12.4	9.5
40–44	24.8	20.4	15.0
45–49	33.7	32.0	24.6
50–54	37.1	35.6	28.6
55–59	55.5	52.0	39.6
60–64	62.5	57.0	51.0
65–69	78.5	71.1	61.0
70–74	103.5	86.2	88.0
75–79	113.5	107.4	103.0
80–84	131.1	131.0	112.3
85–	172.5	122.5	122.2

TABLE 7 — MALES

Capital	Provincial Towns	Rural Areas
2.0	2.1	1.3
2.4	3.0	2.8
5.2	6.2	3.6
8.5	7.9	5.7
15.8	12.6	9.8
24.8	18.3	18.3
45.8	27.2	23.8
60.3	45.9	37.7
86.6	64.9	54.7
127.6	95.0	79.4
154.7	113.6	98.9
178.3	164.6	124.0
204.8	122.2	145.5

DANISH CANCER REGISTRY: 1942 TO 1944
CANCER MORBIDITY PER 10,000 LIVING
(Cancer of Inaccessible Sites, Including Stomach)

TABLE 3 — FEMALES

Age	Capital	Provincial Towns	Rural Areas
25–29	0.5	1.0	1.0
30–34	1.7	1.2	1.8
35–39	3.2	3.9	3.0
40–44	5.1	5.6	4.8
45–49	8.7	9.5	9.0
50–54	11.7	11.7	13.8
55–59	22.6	22.2	20.7
60–64	28.7	31.3	31.1
65–69	39.8	42.0	45.1
70–74	65.9	62.4	74.0
75–79	80.6	96.3	97.5
80–84	101.5	117.0	113.6
85–	112.8	111.1	118.5

TABLE 8 — MALES

Capital	Provincial Towns	Rural Areas
0.9	0.9	0.7
1.6	1.6	1.5
2.8	3.7	1.9
6.4	4.9	3.9
11.7	10.2	8.1
18.4	14.5	15.0
35.7	25.2	21.8
50.2	38.5	34.5
66.7	63.4	55.0
102.3	91.9	82.8
134.0	109.6	100.0
160.9	138.4	112.4
184.7	104.4	99.5

DANISH CANCER REGISTRY: 1942 TO 1944
CANCER MORBIDITY PER 10,000 LIVING
(Cancer of Inaccessible Sites, Stomach Excluded)

TABLE 4 — FEMALES

Age	Capital	Provincial Towns	Rural Areas
25–29	0.5	0.8	1.0
30–34	1.2	0.9	1.6
35–39	2.9	2.7	2.5
40–44	4.4	4.9	3.6
45–49	7.2	7.7	6.9
50–54	10.1	8.8	9.6
55–59	18.9	16.1	14.5
60–64	22.4	21.1	20.2
65–69	26.9	23.8	25.8
70–74	42.4	32.5	41.6
75–79	50.4	48.2	51.0
80–84	58.5	61.2	51.3
85–	66.3	55.6	52.8

TABLE 9 — MALES

Capital	Provincial Towns	Rural Areas
0.8	0.8	0.6
1.2	1.6	1.2
2.2	3.2	1.4
4.8	3.3	2.4
10.1	7.1	4.9
14.0	8.6	9.2
28.3	16.0	12.2
35.2	22.4	17.8
47.0	34.4	27.6
75.4	52.6	44.4
88.7	53.5	49.3
100.0	86.9	57.3
124.5	62.2	56.3

DANISH CANCER REGISTRY: 1942 TO 1944
CANCER MORBIDITY PER 10,000 LIVING
(Cancer of Accessible Sites)

TABLE 5

FEMALES

Age	Capital	Provincial Towns	Rural Areas
25–29	2.3	2.2	1.9
30–34	4.9	6.6	5.0
35–39	10.4	9.7	7.0
40–44	20.4	15.5	11.3
45–49	26.5	24.3	17.7
50–54	27.0	26.9	19.0
55–59	36.6	35.9	25.1
60–64	40.1	35.9	30.8
65–69	51.6	47.3	35.3
70–74	61.1	53.7	46.5
75–79	63.1	59.3	52.0
80–84	72.6	69.8	61.0
85–	106.1	67.0	69.4

TABLE 10

MALES

Age	Capital	Provincial Towns	Rural Areas
25–29	1.2	1.3	0.7
30–34	1.2	1.4	1.6
35–39	3.0	3.1	2.2
40–44	3.7	4.6	3.3
45–49	5.7	5.4	5.0
50–54	10.8	9.7	9.1
55–59	17.5	11.2	11.6
60–64	25.1	23.5	19.8
65–69	39.6	30.5	27.1
70–74	52.3	42.4	35.0
75–79	66.0	60.1	49.6
80–84	78.3	77.8	66.7
85–	80.3	60.0	89.2

TABLE 1A

TEST OF SIGNIFICANCE:* FEMALES
MORBIDITY 1942 TO 1944
(Cancer of All Sites, Including Stomach)

Age	cap.-pr.t.	pr.t.-r.a.	cap.-r.a.†
25–29	−0.73	0.63	−0.17
30–34	−1.56	1.06	−0.28
35–39	−0.02	2.86⊕⊕	2.91⊕⊕
40–44	2.12⊕	3.10⊕⊕	5.65⊕⊕⊕
45–49	0.53	3.34⊕⊕⊕	4.04⊕⊕⊕
50–54	0.07	2.28⊕	2.42⊕
55–59	0.29	3.81⊕⊕⊕	4.26⊕⊕⊕
60–64	0.37	1.32	1.80
65–69	0.37	1.78	2.24⊕
70–74	1.37	−0.62	0.93
75–79	−1.08	0.62	−0.60
80–84	−0.77	0.83	−0.03
85–	1.60	−0.46	1.36

TABLE 2A

TEST OF SIGNIFICANCE: FEMALES
MORBIDITY 1942 TO 1944
(Cancer of All Sites,
Stomach Excluded)

cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
−0.50	0.38	−0.17
−1.73	0.98	−0.54
0.52	2.44⊕	3.17⊕⊕
2.18⊕	3.46⊕⊕⊕	6.10⊕⊕⊕
0.67	3.53⊕⊕⊕	4.41⊕⊕⊕
0.50	2.93⊕⊕	3.61⊕⊕⊕
0.94	4.10⊕⊕⊕	5.33⊕⊕⊕
1.30	1.66	5.22⊕⊕⊕
1.41	2.28⊕	3.95⊕⊕⊕
2.48⊕	−0.30	2.51⊕
0.64	0.54	1.28
0.01	1.55	1.58
2.27⊕	0.02	2.62⊕⊕

TABLE 3A

TEST OF SIGNIFICANCE: FEMALES
MORBIDITY 1942 TO 1944
(Cancer of Inaccessible Sites,
Including Stomach)

Age	cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
25–29	−1.50	0.11	−1.52
30–34	1.42	−1.42	−0.27
35–39	−0.91	1.36	0.34
40–44	−0.55	0.94	0.33
45–49	−0.58	0.47	−0.18
50–54	0.04	−1.37	−1.38
55–59	0.15	0.72	0.85
60–64	−0.84	0.05	−0.90
65–69	−0.56	−0.87	−1.52
70–74	0.60	−2.16⊕	−1.52
75–79	−1.86	−0.15	−2.26⊕
80–84	−1.21	0.29	−1.08
85–	0.09	−0.43	−0.33

TABLE 4A

TEST OF SIGNIFICANCE: FEMALES
MORBIDITY 1942 TO 1944
(Cancer of Inaccessible Sites,
Stomach Excluded)

cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
−1.06	−0.35	−1.52
1.30	−1.80	−0.82
0.26	0.46	0.78
−0.53	1.60	1.04
−0.36	0.69	0.29
0.89	−0.65	0.36
1.29	0.87	2.41⊕
0.51	0.41	1.00
1.02	−0.74	0.41
2.24⊕	−2.28⊕	0.21
0.36	−0.51	−0.11
−0.29	1.20	0.90
0.76	0.24	1.10

* and † See corresponding footnotes to Table 1A, paper IV.

TABLE 5A

TEST OF SIGNIFICANCE: FEMALES
MORBIDITY 1942 TO 1944
(Cancer of Accessible Sites)

AGE	cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
25–29	0.02	0.68	0.72
30–34	−2.96⊕⊕	1.92	−0.17
35–39	0.52	2.54⊕	3.21⊕⊕
40–44	2.72⊕⊕	3.07⊕⊕	6.33⊕⊕⊕
45–49	0.97	3.70⊕⊕⊕	4.93⊕⊕⊕
50–54	0.06	3.90⊕⊕⊕	4.09⊕⊕⊕
55–59	0.25	4.38⊕⊕⊕	4.80⊕⊕⊕
60–64	1.24	1.80	3.31⊕⊕⊕
65–69	1.00	3.48⊕⊕⊕	4.71⊕⊕⊕
70–74	1.36	1.59	3.18⊕⊕
75–79	0.55	1.22	1.87
80–84	0.27	1.00	1.32
85–	2.32⊕	−0.19	2.48⊕

TABLE 6A

TEST OF SIGNIFICANCE: MALES
MORBIDITY 1942 TO 1944
(Cancer of All Sites, Including Stomach)

cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
−0.08	1.68	1.60
−0.33	−0.18	−0.56
−0.92	3.30⊕⊕⊕	2.19⊕
0.34	2.14⊕	2.54⊕
0.91	1.66	2.74⊕⊕
1.88	0.07	2.28⊕
4.56⊕⊕⊕	1.04	6.49⊕⊕⊕
2.64⊕⊕	1.98⊕	5.08⊕⊕⊕
1.83	2.24⊕	4.36⊕⊕⊕
2.04⊕	2.18⊕	4.44⊕⊕⊕
2.06⊕	1.88	4.12⊕⊕⊕
0.98	2.21⊕	3.08⊕⊕
2.80⊕⊕	−1.02	2.37⊕

TABLE 7A

TEST OF SIGNIFICANCE: MALES
MORBIDITY 1942 TO 1944
(Cancer of All Sites, Stomach Excluded)

AGE	cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
25–29	−0.22	1.84	1.58
30–34	−0.84	0.24	−0.74
35–39	−1.07	3.42⊕⊕⊕	2.13⊕
40–44	0.43	2.19⊕	2.69⊕⊕
45–49	1.80	2.04⊕	4.20⊕⊕⊕
50–54	2.72⊕⊕	−0.01	3.21⊕⊕
55–59	5.62⊕⊕⊕	1.42	8.19⊕⊕⊕
60–64	3.27⊕⊕	2.52⊕	6.39⊕⊕⊕
65–69	3.68⊕⊕⊕	2.36⊕	6.70⊕⊕⊕
70–74	3.74⊕⊕⊕	2.50⊕	6.78⊕⊕⊕
75–79	3.30⊕⊕⊕	1.69	5.42⊕⊕⊕
80–84	0.66	2.85⊕⊕	3.30⊕⊕⊕
85–	2.64⊕⊕	−1.13	2.10⊕

TABLE 8A

TEST OF SIGNIFICANCE: MALES
MORBIDITY 1942 TO 1944
(Cancer of Inaccessible Sites, Including Stomach)

cap.-pr.t.	pr.t.-r.a.	cap. r.a.
0.18	0.48	0.68
−0.14	0.30	0.14
−1.11	3.13⊕⊕	1.78
1.34	1.26	2.88⊕⊕
0.94	1.68	2.80⊕⊕
1.85	−0.26	1.92
3.44⊕⊕⊕	1.49	5.60⊕⊕⊕
2.88⊕⊕	1.30	4.72⊕⊕⊕
0.60	1.95	2.60⊕⊕
1.27	1.45	2.84⊕⊕
2.05⊕	1.10	3.40⊕⊕⊕
1.18	1.94	3.09⊕⊕
2.74⊕⊕	0.28	3.44⊕⊕⊕

TABLE 9A

TEST OF SIGNIFICANCE: MALES
MORBIDITY 1942 TO 1944
(Cancer of Inaccessible Sites, Stomach Excluded)

AGE	cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
25–29	−0.03	0.65	0.62
30–34	−0.84	0.91	−0.08
35–39	−1.37	3.38⊕⊕⊕	1.72
40–44	1.68	1.28	3.30⊕⊕⊕
45–49	2.12⊕	2.32⊕	4.69⊕⊕⊕
50–54	3.12⊕⊕	−0.49	3.22⊕⊕
55–59	4.76⊕⊕⊕	2.20⊕	7.88⊕⊕⊕
60–64	3.96⊕⊕⊕	2.02⊕	6.78⊕⊕⊕
65–69	2.92⊕⊕	2.18⊕	5.64⊕⊕⊕
70–74	3.44⊕⊕⊕	1.78	5.76⊕⊕⊕
75–79	3.90⊕⊕⊕	0.69	5.24⊕⊕⊕
80–84	0.87	2.96⊕⊕	3.66⊕⊕⊕
85–	2.66⊕⊕	0.44	3.51⊕⊕⊕

TABLE 10A

TEST OF SIGNIFICANCE: MALES
MORBIDITY 1942 TO 1944
(Cancer of Accessible Sites)

cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
−0.26	1.86	1.56
−0.34	−0.56	−0.94
−0.18	1.56	1.34
−1.04	1.78	0.54
0.25	0.52	0.82
0.68	0.46	1.26
3.04⊕⊕	−0.30	3.33⊕⊕⊕
0.54	1.55	2.16⊕
2.27⊕	1.14	3.82⊕⊕⊕
1.74	1.76	3.71⊕⊕⊕
0.69	1.68	2.34⊕
0.03	1.09	1.00
0.98	−1.87	−0.44

DANISH CANCER REGISTRY: 1942 TO 1944
CANCER MORBIDITY PER 10,000 LIVING
(Cancer of the Stomach)

TABLE 11

FEMALES

Age	Capital	Provincial Towns	Rural Areas
25–29		0.2	
30–34	0.4	0.3	0.2
35–39	0.3	1.2	0.6
40–44	0.7	0.7	1.2
45–49	1.5	1.9	2.0
50–54	1.7	2.9	4.2
55–59	3.7	6.0	6.2
60–64	6.3	10.2	10.9
65–69	12.9	18.2	19.3
70–74	23.5	29.9	32.4
75–79	30.2	48.2	46.4
80–84	43.0	55.8	62.3
85–	46.4	55.6	65.7

TABLE 12

MALES

Capital	Provincial Towns	Rural Areas
0.2	0.1	0.1
0.4	0.1	0.3
0.6	0.5	0.5
1.6	1.6	1.4
1.6	3.1	3.3
4.4	5.9	5.7
7.4	9.2	9.6
14.9	16.1	16.7
19.7	29.0	27.4
26.9	39.2	38.4
45.3	56.1	50.7
60.9	51.5	55.1
60.2	42.2	43.2

DANISH CANCER REGISTRY: 1942 TO 1944
CANCER MORBIDITY PER 10,000 LIVING
(Cancer colli uteri) (Cancer corpus uteri)

TABLE 13

FEMALES

Age	Capital	Provincial Towns	Rural Areas
25–29	1.2	1.3	1.4
30–34	3.0	3.7	2.7
35–39	5.7	5.1	3.3
40–44	10.3	6.5	4.5
45–49	10.0	8.8	4.4
50–54	8.5	7.8	4.1
55–59	9.4	8.6	2.8
60–64	6.2	5.6	4.2
65–69	5.6	6.1	2.0
70–74	7.1	4.8	2.1
75–79	5.6	3.7	2.1
80–84	4.4	0.8	3.1
85–	6.6	1.6	

TABLE 14

FEMALES

Capital	Provincial Towns	Rural Areas
	0.2	0.1
	0.2	0.3
0.1	0.4	0.5
0.7	0.3	0.6
1.2	2.0	1.7
3.4	3.5	2.1
4.4	3.6	2.7
3.2	3.2	2.6
3.0	4.6	2.4
3.3	4.2	1.5
2.0	2.1	2.1
3.7	2.3	1.3
1.7		0.9

DANISH CANCER REGISTRY: 1942 TO 1944
CANCER MORBIDITY PER 10,000 LIVING
(Cancer uteri unspec.) (Cancer mammae)

TABLE 15

FEMALES

Age	Capital	Provincial Towns	Rural Areas
25–29	0.2	0.2	0.0
30–34	0.1	0.2	0.2
35–39	0.1		0.1
40–44	0.8		0.2
45–49	0.4	0.5	0.2
50–54	0.9	0.7	0.4
55–59	1.4	0.8	0.8
60–64	1.3	0.6	0.9
65–69	3.2	1.7	1.5
70–74		4.0	1.4
75–79	0.8	2.9	3.7
80–84	2.2	1.6	4.4
85–		8.2	5.6

TABLE 16

FEMALES

Capital	Provincial Towns	Rural Areas
0.2	0.2	0.0
0.9	1.6	1.2
3.1	2.1	2.2
6.4	6.4	4.3
9.7	9.2	8.2
9.1	9.4	7.3
13.1	14.5	10.7
18.1	16.4	12.4
22.8	19.5	15.9
23.7	19.0	16.0
23.8	18.9	16.8
20.7	23.3	16.7
43.1	18.0	20.4

DANISH CANCER REGISTRY: 1942 TO 1944
CANCER MORBIDITY PER 10,000 LIVING
(Cancer cutis)

TABLE 17

FEMALES

Age	Capital	Provincial Towns	Rural Areas
25–29	0.4	0.2	
30–34	0.3	0.2	0.3
35–39	0.6	0.9	0.2
40–44	0.6	1.0	0.8
45–49	2.2	1.8	1.2
50–54	1.1	2.3	1.2
55–59	3.2	2.6	2.5
60–64	3.4	3.5	3.6
65–69	5.6	5.6	5.0
70–74	8.6	9.3	8.5
75–79	7.9	13.6	9.4
80–84	16.3	13.2	12.7
85–	24.9	16.3	20.4

TABLE 18

MALES

Capital	Provincial Towns	Rural Areas
0.6	0.3	0.0
0.4	0.3	0.2
0.7	0.4	0.3
1.2	1.3	0.7
1.7	1.5	1.2
3.1	3.4	2.5
4.1	3.3	2.7
4.4	6.1	6.0
6.5	8.3	9.7
9.5	14.3	11.4
17.3	22.2	15.8
15.9	25.2	25.8
12.0	26.7	35.7

TABLE 11A

TEST OF SIGNIFICANCE: FEMALES
MORBIDITY 1942 TO 1944
(Cancer of the Stomach)

AGE	cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
25–29	−1.77	2.04⊕	
30–34	0.87	0.50	1.10
35–39	−2.56⊕	1.94	−1.01
40–44	−0.17	−1.15	−1.37
45–49	−0.59	−0.30	−0.98
50–54	−1.74	−1.56	−3.50⊕⊕⊕
55–59	−2.14⊕	−0.13	−2.54⊕
60–64	−2.45⊕	−0.47	−3.23⊕⊕
65–69	−2.22⊕	−0.48	−2.97⊕⊕
70–74	−1.72	−0.71	−2.65⊕⊕
75–79	−3.21⊕⊕	0.31	−3.28⊕⊕
80–84	−1.49	−0.76	−2.43⊕
85–	−0.70	−0.82	−1.59

TABLE 12A

TEST OF SIGNIFICANCE: MALES
MORBIDITY 1942 TO 1944
(Cancer of the Stomach)

cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
0.54	−0.34	0.30
1.70	−1.54	0.38
0.30	0.19	0.54
−0.12	0.42	0.27
−2.08⊕	−0.29	−2.62⊕⊕
−1.29	0.18	−1.31
−1.15	−0.31	−1.63
−0.50	−0.28	−0.84
−2.78⊕⊕	0.54	−2.69⊕⊕
−2.58⊕⊕	0.19	−2.75⊕⊕
−1.38	0.86	−0.82
0.80	−0.41	0.56
1.02	−0.08	1.10

TABLE 13A

TEST OF SIGNIFICANCE: FEMALES
MORBIDITY 1942 TO 1944
(Cancer colli uteri)

AGE	cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
25–29	−0.18	−0.22	−0.42
30–34	−1.78	1.55	0.54
35–39	0.58	2.56⊕	3.30⊕⊕⊕
40–44	3.12⊕⊕	2.30⊕	6.02⊕⊕⊕
45–49	0.86	4.40⊕⊕⊕	5.50⊕⊕⊕
50–54	0.54	3.66⊕⊕⊕	4.40⊕⊕⊕
55–59	0.55	5.70⊕⊕⊕	6.48⊕⊕⊕
60–64	0.46	1.28	1.86
65–69	−0.39	3.93⊕⊕⊕	3.52⊕⊕⊕
70–74	1.32	2.33⊕	3.90⊕⊕⊕
75–79	0.96	1.23	2.36⊕
80–84	1.91	−1.48	0.66
85–	1.40	1.48	2.94⊕⊕

TABLE 14A

TEST OF SIGNIFICANCE: FEMALES
MORBIDITY 1942 TO 1944
(Cancer corporis uteri)

cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
−1.77	0.48	−1.49
−2.50⊕	−0.44	−2.67⊕⊕
−1.78	−0.32	−2.24⊕
1.38	−1.29	0.24
−1.32	0.50	−1.00
−0.11	2.03⊕	1.96⊕
0.77	1.12	2.06⊕
−0.01	0.64	0.64
−1.31	2.28⊕	0.80
−0.68	2.65⊕⊕	1.89
−0.06	−0.01	−0.08
0.64	0.70	1.46
1.22	−0.98	0.41

TABLE 15A

TEST OF SIGNIFICANCE: FEMALES
MORBIDITY 1942 TO 1944
(Cancer uteri unspec.)

AGE	cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
25–29	−0.07	1.01	0.95
30–34	−1.46	0.32	−0.90
35–39	1.19	−1.33	−0.10
40–44	3.50⊕⊕⊕	−1.65	2.66⊕⊕
45–49	−0.47	1.30	0.77
50–54	0.37	0.87	1.33
55–59	1.02	−0.07	1.13
60–64	1.22	−0.70	0.70
65–69	1.58	0.32	2.18⊕
70–74	−4.75⊕⊕⊕	2.64⊕⊕	−2.99⊕⊕
75–79	−1.76	−0.54	−2.43⊕
80–84	0.40	−1.48	−1.14
85–	−2.68⊕⊕	0.64	−2.40⊕

TABLE 16A

TEST OF SIGNIFICANCE: FEMALES
MORBIDITY 1942 TO 1944
(Cancer mammae)

cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
−0.05	1.45	1.41
−1.80	0.88	−0.74
1.44	−0.18	1.47
0.08	2.40⊕	2.54⊕
0.35	0.83	1.26
−0.20	1.66	1.48
−0.75	2.42⊕	1.61
0.77	2.10⊕	3.06⊕⊕
1.15	1.59	2.95⊕⊕
1.41	1.14	2.78⊕⊕
1.18	0.64	2.00⊕
−0.44	1.36	0.88
2.54⊕	0.34	2.60⊕⊕

TABLE 17A

TEST OF SIGNIFICANCE: FEMALES
MORBIDITY 1942 TO 1944
(Cancer cutis)

AGE	cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
25–29	1.02	2.04⊕	3.83⊕⊕⊕
30–34	1.40	−0.44	−0.11
35–39	−0.92	2.77⊕⊕	1.76
40–44	−1.12	0.72	−0.56
45–49	0.67	1.80	2.64⊕⊕
50–54	−1.92	2.12⊕	−0.09
55–59	0.74	0.13	1.00
60–64	−0.04	−0.10	−0.14
65–69	0.00	0.43	0.44
70–74	−0.31	0.39	0.04
75–79	−1.92	1.55	−0.64
80–84	0.66	0.12	0.88
85–	1.04	−0.58	0.60

TABLE 18A

TEST OF SIGNIFICANCE: MALES
MORBIDITY 1942 TO 1944
(Cancer cutis)

cap.-pr.t.	pr.t.-r.a.	cap.-r.a.
1.26	1.78	3.25⊕⊕
0.32	0.44	0.82
1.21	0.44	1.90
−0.16	1.77	1.57
0.35	0.58	1.00
−0.31	1.12	0.75
0.70	0.81	1.65
−1.20	0.06	−1.34
−1.01	−0.81	−1.93
−1.69	1.25	−0.80
−1.02	1.76	0.41
−1.30	−0.09	−1.53
−1.30	−0.90	−2.10⊕

Observations on the Antiproteolytic Reaction of the Serum of Mice: Strain Variations*

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Variations in the antiproteolytic reaction of the serum have been observed in animals and in human patients. Variations among species have been recorded (1, 2) but within a given species there is little variation in the reaction of normal animals except for the guinea pig in which moderate differences have been noted. So far as we know, no previous studies have been made in mice probably because of their small size and blood volume. That this reaction is of some importance in the response of the animal or patient to disease or other stimuli has been recognized. The relationship of the antiproteolytic reaction of human serum to the presence of malignant tumors (3, 4), make it desirable to extend this study to mice because of the importance of pure strains of mice in cancer research. A preliminary study using blood drawn from the heart and testing the antiproteolytic titre by a method previously reported (4), indicated definite variations among different strains of mice. A modification of the method using small amounts of blood from the tail of the mouse was developed in order to be able to preserve the animal essentially unchanged for repeat examination and to determine the effect of tumors and other agents on the titre.

METHOD

The animal is gently placed and held in a small cage with the tail protruding, and a section just large enough to produce bleeding is cut from the tip of the tail.[1] Blood is expressed in droplets by milking with gentle pressure applied over the tail veins beginning at the proximal end. The blood is collected in a capillary tube 10 to 14 cm. in length with a 2 mm. bore.[1] Approximately 0.08 to 0.1 cc. may be collected before clotting occurs. The end away from the column of blood is then flame-sealed so as not to heat the blood. Serum is obtained by centrifuging the capillary tube held by a cork in a test tube at 1500 r.p.m. for 20 minutes.

The capillary tube is snapped in two at the point of separation of serum and cells and the serum is aspirated into a pipette[2] calibrated to deliver a constant weight of serum, the equivalent volume of which has been determined (5). A pipette which delivers about 35 cu. mm. of serum is used. The serum is delivered at once from the calibrated pipette into a volume of normal saline sufficient to give a $\frac{1}{60}$ dilution. Three-tenth cc. aliquots of this dilution are taken for further dilutions, in increments of 10, to be used in determining the titre of the antiproteolytic reaction. Six such aliquots can be obtained from the amount of serum used, and the dilution must be made in a range in which an end point will be reached, which will vary with the strain tested. The dilutions are then treated as in the routine antiproteolytic test (4). Reconstituted pooled human plasma (PK) is used as a control for daily variations.

Duplicate runs on the same serum gave identical results nine out of ten times, and the maximum variation was one dilution. The titre obtained by this tail blood method was correlated with the titre obtained from heart blood, and the results by the two methods varied no more than one dilution (Table 1). Animals were then tested at intervals of one week and the titres were identical in 66 per cent of tests, with a maximum variation of two dilutions occurring in only 5 per cent of tests. Several animals showed minimal or no variations over a period of three months (Table 2).

RESULTS

Preliminary tests using the heart puncture method were made on over 200 mice of which 62 were normal males of the A, C3H, C57, F, BC, and JK strains.[3] All animals were sacrificed and autopsied, and only those free of recognizable disease are included. These tests revealed definite strain differences with the BC and JK strains having high titres, the F's having low titres and the A, C3H and C57 being intermediate (Fig. 1).

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† Senior Fellow, American Cancer Society, as recommended by the Committee on Growth of the National Research Council.

‡ A portion of the data reported will be used as part of a thesis to be submitted to Yale University School of Medicine by Daniel Elliott as a requirement for the degree of Doctor of Medicine.

[1] Tubes available commercially as melting point capillary tubes.

[2] LinderStrom-Lange Pipette.

[3] We are deeply grateful to Drs. L. C. Strong and W. U. Gardner who supplied us with large numbers of valuable animals.

Using the tail blood method, 184 animals of the A, C3H, C57, F, L, $C_{12}I$, JK, BC and the BL, LCS and BRS sublines of the NHO strain were tested. The results of the first tests on each group are outlined in Figure 2. Repeated tests confirmed the results. That the mice fall into these same three groups with high, intermediate, and low titres is apparent. The JK and BC strains and the NHO sublines are in the group with high titres, the A, C3H, C57 and $C_{12}I$ are in the intermediate group and the F's have low titres. The results with the L strain are only preliminary since only one titre was done on each animal and the number of animals is very small. In every strain studied and also in hybrids the titre of the male is definitely higher than that of the female (Fig. 2, A and B).

DISCUSSION

The distinct differences in the serum antiproteolytic titre of mice of different strains are difficult to explain in view of the slight variations found in heterozygous species. In apparently normal dogs and rabbits, under conditions in this laboratory, the greatest variation in actual titres has been from 40 to 80, and over 80 per cent of the animals have titres of 50 or 60 (unpublished data). Guinea pigs on the other hand vary more, but there are no differences as great as that between the low 50 of the female F mouse and the high 140 of the male BRS mouse. These variations are another indication of the basic strain differences in mice. Similar variations in the serum coagulability, cellular components, and hemoglobin of the blood in several strains have also been reported and correlated with tumor susceptibility (6, 7, 8), as have the strain differences in bio-electric properties (9).

It is tempting to correlate the tumor susceptibility or resistance of these strains of mice with variations in the antiproteolytic titre. A division into high titre (BC and JK strains, and sublines

TABLE 1

SERIES OF TESTS SHOWING CORRELATION BETWEEN TITRES OF HEART BLOOD AND TAIL BLOOD, WHICH REVEALS SIMILARITY OF RESULTS

Strain	Sex	Animal number	Titre; Heart blood	Titre; Heart blood with pipette	Titre; Tail blood with pipette
JK	M	1	130	130	130
	M	2	130	120	120
	F	3	110	100	100
	F	4	100	100	90
LCS	M	5	120	110	120
	M	6	100	100	110
	F	7	80	80	80
	F	8	90	90	100
PBR	M	9	130	130	130
	M	10	130	130	130
	F	11	90	80	90
	F	12		90	90
C57	M	13	110	120	120
	M	14	120	110	120
	F	15	90	90	100
	F	16	100	90	90
C3H	M	17	90	90	100
	M	18	90	100	100
	F	19	70	70	70
	F	20	80	70	80
A	M	21	100	90	
	M	22	90	90	100
	F	23	60	60	70
	F	24	70	70	70
BC	M	25	140		140
	M	26	130		130
	F	27	100		100
	F	28	90		100

TABLE 2

ANIMALS IN WHICH REPEATED TESTS BY TAIL BLOOD METHOD WERE DONE AT INTERVALS OF 1 WEEK OR MORE (ALL ANIMALS IN EACH GROUP ARE RECORDED; OTHER GROUPS GAVE SIMILAR RESULTS)

Strain	Sex	Animal number	Titre; actual over % PK				
			Original	1st week	2d week	3d week	3d month
BRS	male	1	120/240	130/260			
"	"	2	110/220	110/220			
"	"	3	110/220	110/220			
"	"	4	130/260	130/260			
"	"	5	140/280	140/280			
"	"	6	110/220	110/220			
"	"	7	110/220	110/220			
"	"	8	100/200	120/240			
"	"	9	110/220	110/220			
"	"	10	130/260	110/220			
"	"	11	100/200	100/200			
"	"	12	110/220	120/240			
C57	male	1	100/183	90/180	90/180	90/180	other treatment
"	"	2	100/183	110/220	110/220	110/200	90/180
"	"	3	100/200	100/200	90/180	90/180	90/180
"	"	34	100/200	100/200	100/200	100/200	other treatment
A	female	31	70/140		70/140	70/140	70/140
"	"	34	70/140		70/140	70/140	60/120

BRS, B1 and LCS of the Strong NHO strain), intermediate titre (A, C3H, C57, and $C_{12}I$) and low titre (F) strains can be made. It is of note that in general it is the intermediate group which is most susceptible to methylcholanthrene-induced[4] and spontaneous tumors, while the high titre strains are more resistant to MC induced tumors and rarely develop spontaneous tumors except for the NHO strain. The F strain with a very low titre is the most resistant to methylcholanthrene-induced tumors. This strain (F) has a high incidence of leukemia (10) and is unusual in many respects, being difficult to breed, subject to many diseases and extremely sensitive to physical trauma.

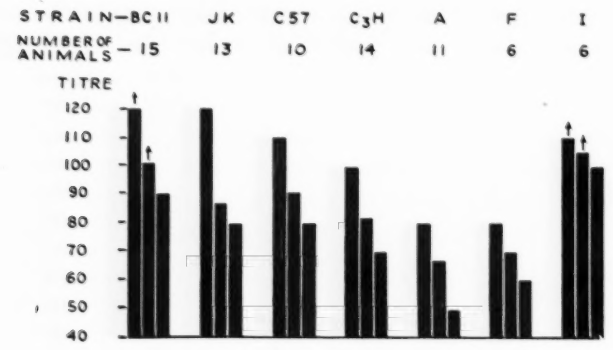
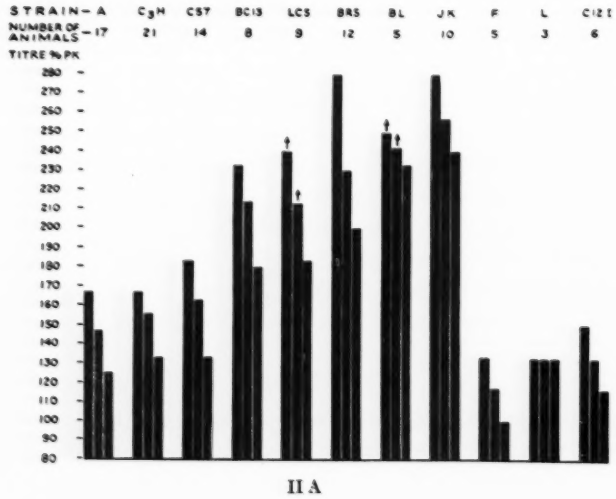
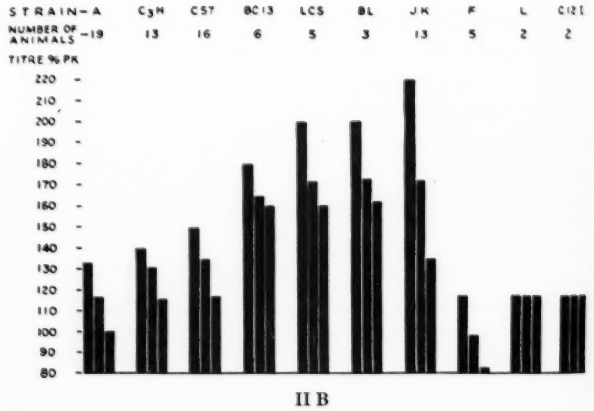


FIG. 1.—Columnar graphs indicating the highest, lowest, and mean titres in male mice of 7 strains. Blood obtained by heart puncture. Strain and number of animals in each group appear at top of figure.



II A



II B

FIG. 2.—Columnar graphs indicating ratio of titre of male and female mice of various strains to plasma control titre (PK). Blood obtained from tail of mice. Strain and number of animals in each group appear at tops of figures. Figure 2A—males, Figure 2B—females.

No satisfactory explanation for the variations of antiproteolytic titre is different strains of mice has been found and indeed little is known about the basic nature or method of formation of this material. Since many of these strains of mice are being studied exhaustively from all points of view, they should be excellent animals for detailed study of this reaction. Further study of their response to various drugs, trauma, and tumor growth is in progress.

[4] Unpublished data of Dr. L. C. Strong.

SUMMARY

1. A method for determining the antiproteolytic activity of serum of mice is described.

2. Variations have been found in the antiproteolytic activity of the serum in several strains of mice.

3. The possible correlation between these variations and susceptibility to tumor formation is discussed.

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Natural and Immune Antibodies in Mice of Low and High Tumor Strains*

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I. NATURAL HETEROAGGLUTININS FOR SHEEP RED CELLS AND HUMAN RED CELLS IN TUMOR-FREE ANIMALS

In a previous communication striking differences in the distribution of antisheep agglutinins in the serum of mice belonging to four inbred strains were reported (6). Additional investigations on this phenomenon and extension of the study to two more inbred strains are the subject of the first part of the present report.

Material.—The following healthy stock animals were tested for natural antisheep agglutinins: 117 C57 Black (68 males and 49 females); 80 C3H (28 males and 52 females); 132 dba (38 males and 93 females); 56 Marsh-albino (23 males and 33 females); 60 Bagg albino C (34 males and 26 females); 62 Akm (30 males and 32 females). Most animals were from 3 to 8 months old; the age of the others ranged from 10 weeks to 20 months.[1]

Technic.—The animals were sacrificed by decapitation and the blood collected from the severed vessels. The inactivation of the serum, the setting up and the reading of the agglutination tests were reported previously (6).

Results.—The distribution of the antisheep agglutinins is summarized in Table 1. Marked differences were noted between the C57 Black strain, on the one hand, and the other five strains, on the other: in the C57 Black strain, antisheep agglutinins were absent only in 3.5 per cent, whereas they were absent in 67.5 per cent of the C3H, in 45.4 per cent of the dba, in 37.5 per cent of the Marsh-albino, in 35.0 per cent of the B alb C,

TABLE 1

DISTRIBUTION OF NATURAL ANTISHEEP AGGLUTININS IN SIX INBRED MOUSE STRAINS

Titer	Strain											
	C57 Black		C3H		dba		Marsh-albino		B alb C		Akm	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
0	4	3.5	54	67.5	60	45.4	21	37.5	21	35.0	38	61.3
1	2	1.7	13	16.3	25	18.9	6	10.7	15	25.0	8	12.9
2	12	10.3	5	6.2	24	18.2	15	26.8	14	23.3	6	9.7
4	10	8.6	6	7.5	10	7.6	10	17.9	6	10.0	8	12.9
8	24	20.5	2	2.5	6	4.6	3	5.3	4	6.7	2	3.2
Total per cent		44.6		100.0		94.7		98.2		100.0		100.0
16	14	11.9	0		2	1.5	1	1.8	0		0	
32	9	16.2	0		4	3.0	0		0		0	
64	16	13.7	0		1	0.8	0		0		0	
128	7	6.0	0		0		0		0		0	
256	5	4.2	0		0		0		0		0	
512	2	1.7	0		0		0		0		0	
1024	2	1.7	0		0		0		0		0	
Total per cent		55.4				5.3		1.8				
Total no. of animals	117		80		132		56		60		62	

* Presented at the fortieth annual meeting of the American Association for Cancer Research at Detroit, Michigan, on April 16, 1949.

[1] The animals of the C57B, C3H and dba strains had been either bred in this laboratory from parent stock received 3 years ago from Dr. A. Tannenbaum, Michael Reese Hospital, Chicago, or were received from him directly; the animals of the Marsh-albino strain had been bred for 1 year in this laboratory from parent stock received from Dr. S. G. Warner, Biological Station, Springville, N.Y. The animals of the B alb C strain were obtained from the Jackson Memorial Laboratory, Bar Harbor, Maine; the animals of the Akm strain from Carworth Farms, New City, New York.

and in 61.3 per cent of the Akm strains. Furthermore, titers of 16 or more were found in 55.4 per cent of the C57 Black animals, in none of the C3H mice, in 5.3 per cent of dba animals, in 1.8 per cent of the Marsh-albinos and in none of the B alb C and Akm animals. There was no apparent relation between sex, age, and the presence and titer of the natural antisheep agglutinins.

Inactivation (30 minutes at 56° C.), which was routinely used in our tests, had no effect on the titer of the antisheep agglutinins, as shown by parallel tests with not inactivated and inactivated samples of the same serum. As mentioned in the earlier report (6), absorption of the serum with suspensions of boiled guinea pig kidney antigen and with boiled beef red cells failed to remove significant amounts of the agglutinins.

Table 2 presents the effect of bovine albumin or mouse serum used as diluents for the tested serum and the sheep cell suspension, instead of physiologic solution of sodium chloride. The serums used as diluents had been inactivated and freed of their antisheep agglutinins by absorption with sheep red cells. The replacement of the saline by serum or albumin lowered the titer in most instances, or left it unchanged.

Natural antisheep hemolysins.—Tests for these natural antibodies were performed in 8 C57B, 6 C3H, 11 Bagg albino C, and 11 Akm animals. The technic was the same as will be described in part III of this communication. In no instances were antisheep hemolysins detectable, even in undiluted inactivated and not inactivated serum.

Natural agglutinins for human red cells.—Table 3 summarizes the tests for agglutinins for Rh-positive human red cells of group O. They were found only in the minority of serums tested, and mostly in titers of 1 and 2, with rare instances of titers of 4, 8, and 16. No significant difference was noted in their occurrence in the six strains.

A small number of tests using also human red cells of group A and of group B showed similar results, though agglutination of A cells tended to proceed to somewhat higher titers than that of group O cells. No relation was noted between the presence and height of titer of antisheep agglutinins and presence and titer of antihuman agglutinins.

Discussion

The observations on the presence and quantity of natural antisheep agglutinins in the serum of mice of the different inbred strains obviously set apart the C57 Black strain from the other five strains. In Table 4 an attempt was made to correlate the findings pertaining to the antisheep agglutinins with the spontaneous tumor incidence recorded for these strains. These findings suggest that in the strains examined there is an inverse relationship between the susceptibility to spontaneous tumor formation and the ability to produce natural antisheep agglutinins.

The earlier report (6) was confined to strains characterized mainly by a high incidence of mammary carcinoma (C3H, dba, Marsh-albino). In the present work similar relations were noted in the B alb C and the Akm strains, in which tumors other than mammary carcinoma occur frequently. In this connection, it is of interest to recall a study by Andervont (1) in which he investigated the effect of foster-nursing by C3H mothers on the tumor incidence in C57 Black and B alb C mice. While the spontaneous mammary cancer incidence in females nursed by their own mothers was about equally low (3 per cent or less) in both strains, foster-nursing by the high-mammary-cancer strain mothers (C3H) raised the mammary cancer incidence in the B alb C animals to 64 per cent but in

TABLE 2

EFFECT OF DILUENT ON NATURAL ANTISHEEP AGGLUTININS

Strain	No.	Titer		
		Saline	Mouse serum	Bovine albumin
C57B	1	64	8	8
C57B	2	32	4	4
C57B	3	16	4	1
C57B	4	2	1	
C57B	5	2	0	
C57B	6	0	0	0
C57B	PI*	64		16
C57B	PII*	32		32
C57B	PIII*	32		32
C3H	1	0	0	0
C3H	2	1		0
C3H	3	2	1	1
C3H	4	0		0
C3H	5	0		0

* Pooled serum.

TABLE 3

NATURAL AGGLUTININS FOR HUMAN O Rh-POSITIVE RED CELLS IN MOUSE SERUM

Titer	Strain					
	C57B	C3H	dba	Marsh-albino	B alb C	Akm
0	45 (77.6%)	47 (77.0%)	35 (87.5%)	14 (73.6%)	17 (70.8%)	22 (66.7%)
1	7	10	5	4	5	7
2	2	3	0	1	2	4
4	1	1	0	0	0	0
8	2	0	0	0	0	0
16	1	0	0	0	0	0
Total no. of animals	58	61	40	19	24	33

C57 Black animals to only 10 per cent. This was interpreted as showing that the B alb C animals lack the milk factor but in its presence are highly susceptible to development of mammary cancer; on the other hand, C57 Black not only lack the milk factor but remained refractory in 90 per cent even after the milk factor was supplied. For the latter phenomenon, genetic factors may be responsible. It is hoped that the present investigations will be extended to the determination of antisheep agglutinins in animals of high mammary cancer strains which lack the milk factor. Horsorb them differentiates them from the antisheep agglutinins found in infectious mononucleosis (7).

The position of the mouse in the Forssman antigen-antibody system has been the subject of controversial statements (3). More recently, extensive studies of antisheep hemolysins produced in rabbits by injection of various mouse tissues were reported by Brown (2); on the basis of the behavior of these antibodies in absorption experiments and because of other properties, the author concluded that the antigens present in mouse tissues are not identical with the Forssman antigen.

TABLE 4

ANTISHEEP AGGLUTININS AND INCIDENCE OF SPONTANEOUS TUMORS IN SIX INBRED STRAINS OF MICE

		Spontaneous Tumor Incidence in per cent		Antisheep Agglutinins	
Strain	Reference	Mammary ca.	Other tumors	Present (per cent)	Titer 1:16 and higher (per cent)
C57	Little, Murray & Cloudman ('39)	0.5		96.5	55.4
	Haagensen & Randall ('42)	1.1			
	Snell ('41)		Non-epithelial, 10–20		
C3H	Andervont ('41)	91.4		32.5	0
	Bittner ('43)	92.3			
	Heston ('45)		Hepatomas: males, 25 females, 10 Pulmonary: 5–10		
dba	Korteweg ('36)	76.3			
	Murray & Hoffman ('41)	64.5		54.6	5.3
Marsh-albino	Murray & Hoffman ('41)	76.4		62.5	1.8
	Haagensen & Randall ('42)	76.4			
Bagg albino C	Shimkin & Andervont ('42)	1.1		65.0	0
	Andervont ('40)		Pulmonary: 20–30 (also other internal)		
Akm	Law ('48)		Leukemia: 60–80	38.7	0

Data on spontaneous tumors from Walter E. Heston: Genetics of Mammary Tumors in Mice, in "A Symposium on Mammary Tumors in Mice," AAAS, 1945; p. 61, Table I; George D. Snell: Biology of the Laboratory Mouse, The Blakiston Co., Philadelphia, 1941; L. W. Law: Mouse Genetics News No. 2, J. Heredity, 39: 300, 1948.

monal influences can hardly be suspected of being operative in the phenomenon in question, since it is known that male animals of high mammary cancer strains develop mammary cancer only if supplied with estrogens. Since no sex difference was noted in the occurrence of antisheep agglutinins, the conclusion seems justified that hormonal influences are not a factor in the distribution of natural antisheep agglutinins.

Antisheep agglutinins in some species have been found to be of the Forssman type. Hence it was deemed necessary to test the nature of the agglutinins found in our material. They did not react with the guinea-pig kidney antigen; therefore, the conclusion is permissible that they are not of the Forssman type. Failure of beef red cells to absorb them differentiates them from the antisheep agglutinins found in infectious mononucleosis (7).

These conclusions held true for all the inbred strains of mice that were studied. Preliminary work which will be reported elsewhere, so far showed that tissues of mice were capable of stimulating production of antisheep agglutinins and hemolysins in rabbits, but that there was no antigenic difference in the tissues from different inbred strains.

Gorer (8 to 10) reported the existence of antigenic differences between inbred strains of mice. The presence of these antigens, which were found in red blood cells and in tumor tissue, was determined by genetic factors. The transplantability of the tumor was dependent on the presence in the tumor tissues and in the tissues of the host of the identical antigens. In the absence of the homolo-

gous antigens in the tissues of the host, the transplant regressed simultaneously with the development of isoagglutinins for the red cells of the strain in which the tumor had originally arisen (10). Thus far we have not established a relationship with regard to antisheep agglutinins similar to the observations of Gorer regarding isoagglutinins in mice. Further studies are needed regarding any possible relation between antigenic composition of tissues of inbred strains of mice and the distribution of antisheep agglutinins. This subject is being investigated.

On the basis of data available at present, a tentative explanation is proposed which attributes the low incidence and low quantities of these natural antibodies in strains with high tumor susceptibility to an inferior reticulo-endothelial activity in these animals. A morphologic difference, expressed in impaired storage of carmine, has been demonstrated previously for reticulo-endothelial tissue in mice of the C3H and Marsh-albino strains, as compared with the findings in C57 Black animals (14). Also, low titers of natural antibodies, namely of human isoagglutinins, have been observed in patients with chronic leukemia (4). It is realized that the validity of the hypothesis must be checked on additional inbred strains of mice with low susceptibility to spontaneous tumor incidence, of which up to now only a single one (C57 Black) has been available for study.

II. NATURAL ANTISHEEP AGGLUTININS IN TUMOR-BEARING ANIMALS

Material and technic.—The study included 58 C57 Black animals; 41 had subcutaneous sarcomas induced by injection of methylcholanthrene, 17 had transplanted spindle cell sarcomas originally produced by methylcholanthrene and carried by subcutaneous transplants. In C3H animals, the study included 22 bearers of spontaneous mammary carcinoma, 26 mice with transplanted mammary carcinoma, and 23 animals with subcutaneous sarcomas induced by methylcholanthrene. The animals were sacrificed when the tumors had reached weights of from 1 to 3 grams. The same technics were used as in the work with tumor-free animals.

Results.—The findings in C57 Black animals are summarized in Table 5. On the whole, the incidence and the titers of the antisheep agglutinins appeared to follow a similar pattern in tumor-free and tumor-bearing animals. There was a slight increase of titers of 16 and more in the tumor-bearing group as compared with the tumor-free group, the percentage being 74.1 for the former, and 55.4 for the latter. The probable error of difference for these values was calculated as ± 5.0; since the difference between the percentages (18.7) is more than three times the probable error, statistical significance may be ascribed to the difference.

In Table 6 tumor-bearing and tumor-free animals of the C3H strain are compared with each other. None of the animals showed a titer higher than 8. However, the number of animals with agglutinins was increased among those bearing transplanted or induced tumors; it had risen to 63.3 per cent from 32.5 per cent in tumor-free animals. This difference of 30.8 is more than 5 times the probable error of difference (± 5.9) and hence statistically significant. It should be noted that no such rise in the presence of agglutinins was observed among the animals with spontaneous mammary carcinoma, among which only 4 out of 22 possessed the antibodies. The antisheep agglutinins in tumor-bearing animals behaved similarly to those in tumor-free animals with regard to guinea-pig kidney and beef cell antigens, and in relation to various diluents.

TABLE 5

NATURAL ANTISHEEP AGGLUTININS IN TUMOR-BEARING C57 BLACK ANIMALS

	Tumor-free animals		Tumor animals			
			Induced	Transplanted	Total	
Titer	No.	%	sarcomas	sarcomas	No.	%
0	4	3.5	2	1	3	5.2
1–8	48	41.1	5	7	12	20.7
Total per cent		44.6				25.9
16–1024	65	55.4	34	9	43	74.1
Total no. of animals	117		41	17	58	

TABLE 6

NATURAL ANTISHEEP AGGLUTININS IN TUMOR-BEARING C3H ANIMALS

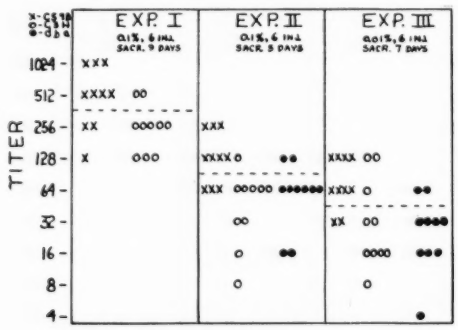
			Tumor Animals					
			(1) Spont. mammary ca.		(2) Transpl. mammary ca.	(3) Induced sarcoma	(2) + (3)	
	Tumor-free Animals							
Titer	No.	%	No.	%	No.	No.	No.	%
0	54	67.5	18	81.8	9	9	18	36.7
1–8	26	32.5	4	18.2	17	14	31	63.3
Total no. of animals	80		22		26	23	49	

Discussion

On the whole, the presence of tumors did not affect the difference in the distribution of antisheep agglutinins which has been exhibited by the tumor-free animals of the low-cancer strain C57 Black and the high-cancer strain C3H. In both strains,

however, the presence of transplanted and induced tumors appeared to favor the production of the antisheep agglutinins. In C57 Black animals, this was expressed in a slightly higher frequency of the elevated titers; in C3H animals, it led to a definite increase in the percentage of animals with agglutinins, although their titer was not elevated. It might be significant that no such increase in the presence of agglutinins was found in C3H animals with spontaneous mammary cancer. One might speculate on the possibility that transplanted and induced tumors are accompanied by certain defensive reactions in the host, futile though they prove eventually, and that this is also expressed in an increased production of the natural antibodies. No such stimulation apparently occurred in animals in whom spontaneous tumors developed.

CHART I



IMMUNE ANTISHEEP AGGLUTININS

However, the rather small number of tumor animals examined to date precludes the drawing of any far-reaching conclusions from these results.

III. IMMUNE AGGLUTININS AND HEMOLYSINS FOR SHEEP AND HUMAN RED CELLS

This part of the study was originally conceived as the primary objective, inasmuch as it was thought that the response of animals to the introduction of an antigen might well serve as an indicator of reticulo-endothelial activity. Only tumor-free animals have been used for this purpose so far. The extensive literature on the possible relationship between the reticulo-endothelial system and neoplasia (15) contains mainly work carried out on tumor-bearing animals or human patients. Hence in all these instances the objection is possible that changes of reticulo-endothelial activity observed under these conditions are secondary effects of the disease rather than primarily connected with it. Therefore, it was decided to use healthy animals in whom it is possible to predict the development of a spontaneous tumor later in life with fair accuracy.

Immune Antisheep Agglutinins

Material.—Healthy, 3 to 6 months old, male and female C57 Black, C3H and dba animals were used. In each experiment, animals of the different strains were paired as to age, sex, and approximate weight.

Technic.—Washed sheep cells were suspended in saline to the desired concentration, and intraperitoneal injections of the suspensions were given on alternate days simultaneously to all animals of each experiment. The concentration of the sheep cell suspension, the dosage and number of injections, and the time interval between the last injection and sacrifice of the animals varied in the individual experiments, and will be listed in the proper place.

The blood was collected from the animals in the same way as in the work on natural antibodies, and the agglutination tests were set up and read as mentioned previously, except that higher dilutions were prepared in order to reach the end point.

Results.—Preliminary experiments were carried out with 50 per cent suspensions of sheep cells. Group I: 11 C57 Black and 13 C3H animals received within 8 days 4 intraperitoneal injections each of 0.1 cc. of the suspension; they were sacrificed 7 days after the last injection. The antisheep agglutinins ranged from 64 to 2048; there was no significant difference of the antibody level in the two strains. Group II: 6 C57 Black and 4 C3H received one single injection of the sheep cell suspension, and were sacrificed 7 days later. Titers of 64 to 256 were obtained in both strains.

In the following 3 experiments, recorded in Chart I, lower dosage of the antigen was used.

Experiment I. One-tenth cc. of a 0.1 per cent saline suspension of sheep cells was injected three times weekly on alternate days for 2 weeks; total injections: 6. The animals were sacrificed 9 days after the last injection. Seven of the 10 C57 Black, but only 2 of the 10 C3H animals showed titers of 512 or higher.

Experiment II. One-tenth cc. of a 0.1 per cent sheep cell suspension was injected as above for a total of 6 injections; the animals were sacrificed 5 days after the last injection. Seven of the 10 C57 Black, but only 1 of the 10 C3H, and 2 of the 10 dba animals reached titers of 128 or more.

Experiment III. One-tenth cc. of a 0.01 per cent sheep cell suspension was injected 6 times on alternate days within 2 weeks. The animals were sacrificed 7 days after the last injection. Eight of 10 C57 Blacks, but only 3 of 10 C3H, and 2 of 10 dba animals showed titers of 64 or more.

Properties of immune antisheep agglutinins.—

Table 7 contains some of the experiments on absorption of the immune agglutinins by guinea-pig kidney and beef cells. Absorption with kaolin in 20 per cent suspension was about equally effective in removing a small amount of the antibodies as were the guinea-pig kidney or beef cell suspensions; hence the absorption by the latter substances was nonspecific. In Table 8 some experiments are recorded in which the effect of bovine albumin and of mouse serum used as diluents instead of saline was studied. Serum used as diluent was first inactivated and sheep agglutinins, if present, were removed by absorption. In analogy with the observations on natural antisheep agglutinins, bovine albumin (20 per cent solution) depressed in most instances the titer found in saline. The effect of mouse serum was irregular and is in need of further investigation. Rat serum (not recorded in the table) had no significant effect on the titer.

Comparisons of agglutinin titers in inactivated and not inactivated samples of the same serums were done in 6 instances. No significant difference between the samples was observed.

Preservation of serums for 3 weeks left the agglutinin titers unchanged in 2 out of 6 cases; in 3 serums the titer dropped by 1 tube, in 1 serum by 2 tubes. This conforms with the findings obtained in natural antisheep agglutinins (6).

Immune Sheep Hemolysin

Material.—In 58 animals, used for experiments on immune antisheep agglutinins, sheep hemolysins also were determined.

Technic.—The serum was inactivated at 56° C. for 30 minutes. Two per cent suspensions of sheep red cells were prepared from washed packed cells. The complement used was either frozen pooled guinea-pig serum, or a lyophilized commercial preparation. The technic of complement titration used in the Kolmer complement fixation test was used (5). However, the final dilution of the complement was carried out in such a manner that two full units were contained in 0.5 cc. of the dilution. Example: the titration showed the first sparkling hemolysis with 0.45 cc. of a 1:30 dilution of the complement. Hence two full units were represented by 1 cc. of this dilution; dilution of the complement to 1:15 resulted in the presence of two full units in 0.5 cc.

The actual test was set up in the following manner: In a row of 10 to 12 test tubes, 5 drops of saline were placed into each tube except the first and second. To the first tube 5 drops of the serum to be tested were added, to the second tube, 2 drops of the serum and 8 drops of saline. After mixing the contents of the second tube, 5 drops were transferred into the third tube, and the transfer continued in the same manner. Serial dilutions were thus obtained starting with 5 in the second tube, 10 in the third, and so forth. To each tube, 5 drops of the sheep cell suspension and 10 drops of complement were then added. The usual controls with complement and saline were set up. Following incubation for one hour in the water bath at 37° C. the tubes were centrifuged, unless complete hemolysis was visible. The degree of hemolysis was recorded as (1) complete, (2) almost complete, when tiny remnants of sediment were present, (3) partial, when marked hemolysis accompanied a residue of cell sediment, (4) slight, when distinct hemolysis of the supernatant was present without marked reduction in the quantity of the sediment, and (5) negative. The titer of each serum was defined both by the highest dilution in which complete hemolysis occurred, and by the highest dilu-

TABLE 7

ABSORPTION OF IMMUNE ANTISHEEP AGGLUTININS BY GUINEA-PIG KIDNEY AND BEEF CELL ANTIGENS

			Titer after absorption with:		
Strain	No.	Unabsorbed	Guinea-pig kidney	Beef cells	Kaolin
C57B	162d	640	320	160	
	152a	640	80	160	
	166a	320	320	160	
	150a	2560	1280	2560	
	pool	160	40		40
C3H	162a	640	320	160	
	157a	640	320	320	
	160b	40	20		20
dba	16c	160	160	160	
	9b	160	80	160	
	16b	160	80	160	
	pool	20	20		20

TABLE 8

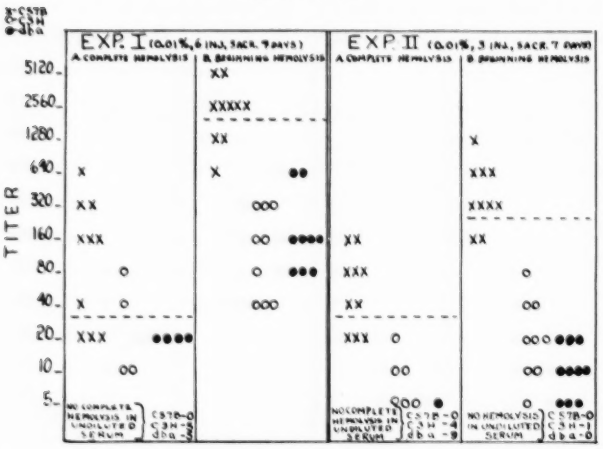
EFFECT OF DILUENT ON IMMUNE ANTISHEEP AGGLUTININS

			Titer	
Strain	No.	Saline	Mouse serum	Bovine albumin
C57B	129a	512		128
	132a	2048		128
	132d	256		32
	140a	1024		64
	172b	128	1024	64
	160c	128	256	16
	167a	64	64	
	179b	16	32	
C3H	M1	128		16
	M3	128		32
	M6	256		32
	M9	512		16
	M12	32	128	16
	155a	256	512	256
	151c	16	8	
	129c	16	8	
dba	M22	64	256	16
	14a	128	128	16
	22a	16	32	

tion in which beginning hemolysis was noted. In the following discussion titers for complete hemolysis and for beginning hemolysis are stated separately.

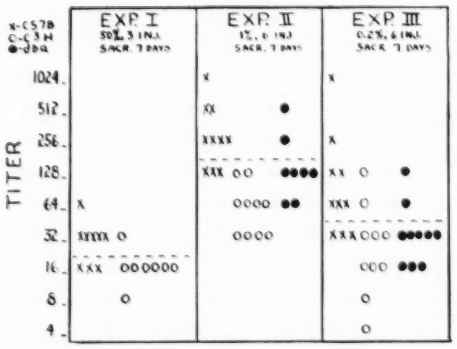
Results.—Experiment I. Ten animals of the C57 Black, 9 animals of the C3H, and 9 animals of the dba strains received on alternate days 6 intraperitoneal injections of 0.1 cc. of a 0.01 per cent sheep cell suspension. The animals were sacrificed 7 days after the last injection.

CHART II



ANTISHEEP HEMOLYSINS

CHART III



IMMUNE AGGLUTININS FOR HUMAN RED CELLS

Chart II summarizes the findings: Complete hemolysis in dilutions of 1:40 or more was found in 7 of 10 C57 Black, but only in 2 of 9 C3H, and in none of 9 dba animals. Beginning hemolysis in titers of 2560 and more was present in 7 of 10 C57 Black, but in none of 9 C3H, and in none of 9 dba animals.

Experiment II. Ten animals each of the C57 Black, C3H, and dba strains received on alternate days a total of three intraperitoneal injections of 0.1 cc. of a 0.01 per cent sheep cell suspension. The animals were sacrificed 7 days after the last injection.

Seven of the 10 C57 Black, but none of the 10 C3H and 10 dba animals showed titers of 40 or more for complete hemolysis. Eight of the 10 C57 Black animals, but none of the dba or C3H animals showed beginning hemolysis at titers of 320 and higher (Chart II).

In both experiments antisheep hemolysins, as well as antisheep agglutinins were determined. Hence it was possible to compare both antibodies in these groups. Although the behavior was not exactly parallel in every detail, the trend toward higher values in agglutinins and hemolysins was similar.

In nine instances serum samples were set up for hemolysin tests using both inactivated and non-inactivated serum portions; the not inactivated samples showed in most instances a rise of the titer by 1 or 2 tubes, as compared with the inactivated samples. This was noted both in regard to beginning and to complete hemolysis.

Immune Agglutinins for Human Red Cells

Material.—Healthy animals of the C57 Black, C3H, and dba strains were used. For each experiment, animals of the different strains were paired as to age and sex.

Technic.—Intraperitoneal injections of suspensions of human Rh-positive red cells of group O were given, the concentration of the suspensions and the number of injections varying in the individual experiments. The animals were sacrificed 7 days after the last injection, the blood collected in the usual manner, and the agglutination tests were performed with the same technic as the one used for antisheep agglutinins. In the first experiment, pooled human cells were used for the injection and titration; in the last 2 experiments the same donor was used for the injections and for the titrations.

Results.—A summary of the experiments is given in Chart III.

Experiment I. Nine C57 Black and 8 C3H animals received on alternate days a total of three intraperitoneal injections of 0.1 cc. of a 50 per cent cell suspension. Six of the 9 C57 Black, but only 1 of the 8 C3H animals showed titers of 32 or more.

Experiment II. Ten C57 Black, 10 C3H, and 8 dba animals received a total of 6 injections of a 1.0 per cent suspension. Seven of the 10 C57 Black, but only 2 of the 8 dba, and none of the C3H animals showed titers of 256 or more.

Experiment III. Ten C57 Black, 10 C3H and 10 dba animals received on alternate days a total of 6 injections of 0.1 cc. of an 0.2 per cent suspension. Seven of the 10 C57 Black, but only 2 of the

10 C3H, and 2 of the 10 dba animals showed titers of 64 or more.

Immune Hemolysins for Human Red Cells

Material.—Animals injected with human red cells, as outlined above, were also tested for hemolysins against human red cells.

Technic.—The same procedure was used as described under immune antisheep hemolysins, except that human red cells of group O were used instead of sheep cells. The complement titration for these experiments was also carried out with sheep cells, because of the fact that amboceptor for human hemolysin was not available.

Results.—Results are summarized in Chart IV.

Experiment I. The animals received 4 injections of the 1.0 per cent suspension of human red cells. Three of the 10 C57 Black, but none of the 10 C3H or 7 dba animals showed complete hemolysis in the undiluted samples. The beginning hemolysis showed titers of 10 or more in all 10 C57 Black, but only in 1 of the 10 C3H and in 1 of the 7 dba animals.

Experiment II. The animals received 6 injections of the 1.0 per cent suspension. Six of the 10 C57 Blacks, but none of the C3H and dba animals showed complete hemolysis at titers of 1 or 5. Beginning hemolysis reached titers of 20 and more in 8 of the 10 C57 Black, but only in 1 of the 10 C3H, and in 1 of the 8 dba animals.

Experiment III. The animals received 6 injections of the 0.2 per cent suspension. All 10 C57 Black animals showed almost complete hemolysis in the undiluted specimen, while none of the C3H and dba animals showed this activity. Beginning hemolysis in titers of 10 and more was present in 8 of the 10 C57 Blacks, but in none of the 10 C3H and of the 10 dba animals.

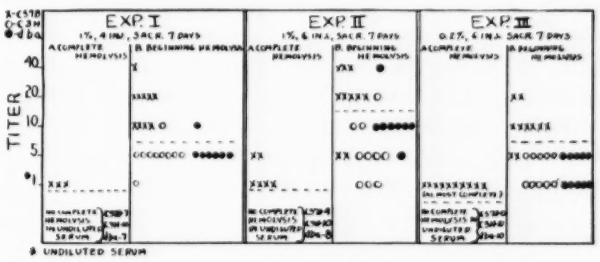
Discussion

Analysis of the material presented under the headings immune sheep agglutinins and hemolysins, and immune human agglutinins and hemolysins clearly demonstrates that C57 Black animals tend to show a better antibody response than animals of the C3H and dba strains which have been treated in the same manner. In the evaluation of the results of the immunization experiments, the highest titer reached by from 70 to 80 per cent of the animals of any one strain was used as the standard of comparison. Only animals of strain C57 Black showed antibody production putting them into this category. This criterion could not be used in the evaluation of experiments determining complete hemolysis of human red cells because of the low titers.

Several points deserve comment. The results of the experiments dealing with immunization with sheep cells are in keeping with the presence of natural antibodies for this antigen, but the qualitatively similar response to immunization with human antigen is the more impressive as natural antibodies for this antigen have been found conspicuously low or absent. It is commonly found that an immunologic response is more pronounced in cases where natural antibodies against the antigen are present than in cases where such natural antibodies are absent.

As shown in the experimental data, the absolute titers of immune antibodies varied greatly with dosage, and concentration of antigen, and time intervals employed. Quite likely, certain of these factors result in an optimum immunologic response while others are less favorable. It appears noteworthy that in the use of sheep cell antigen

CHART IV



IMMUNE HEMOLYSINS FOR HUMAN RED CELLS

very high concentrations (50 per cent suspension) failed to reveal a difference in antibody response between a high and low tumor strain, whereas lower concentrations (0.1 per cent, 0.01 per cent) clearly demonstrated such differences. Also, the absolute titers of antibodies produced by the high dosage were reproduced by use of more frequent injections of a lower concentration. It seems important in studies of this type to select adequate dosage and concentration of antigen, since overdosage may easily lead to inconclusive results.

It is of interest that natural antisheep agglutinins, sometimes found in the C57 Black animals in titers equal to those observed following immunization, are not accompanied by antisheep hemolysins, in contrast to the results obtained in the immunized animals. It will also be noted that in the different experiments there is by no means a sharp dividing line between the individual animals of the different strains, that is, there is a certain overlapping in the immunologic response. This might well be expected in view of the fact that such animals, if left to live out their natural life span, will exhibit different fates in regard to development of, or free-

dom from, tumors. It is regrettable that technical limitations of blood sampling do not permit such a test to be performed with survival and follow-up of the animals. Furthermore, at least in the case of antisheep agglutinins, it is not always possible in the individual animal to be absolutely sure that the agglutinins found are entirely immune in nature, or what part of them might have been preformed prior to immunization. Here again a sampling and testing of blood prior to injection of the antigen would be desirable but has not proved feasible so far.

It is well known that different inbred mouse strains exhibit greatly varying susceptibility to numerous infectious agents; this is utilized in routine work where successful transmission of a virus or other microorganism is desired. In 1938 Gorer and Schütze (12) compared the immunologic response of two heterozygous and two inbred mouse strains, one of which was C57 Black, to the injection of O and H antigens of S. typhi murium and S. enteritidis. They found in C57 Black mice the highest titers of natural agglutinins for strain 0901 of S. typhi murium, but animals of other strains produced higher titers of some immune agglutinins than did C57 Black mice. The authors found no conclusive relationship between antibody production and resistance to infection with the same organism.

Lumsden (13) showed that there exists in rats a relationship between production of isoantibodies and resistance or susceptibility to tumor transplantation. He found that rat erythrocytes contain isoagglutinogens but rat serum normally is free of isoagglutinins. When rats were transplanted with Jensen sarcoma, a certain number of them developed progressive tumor growth while in others the tumors did not take or regressed after an initial period of growth. In the latter animals—resistant to tumor transplantation—isoagglutinins for rat erythrocytes were detectable, while they were not found in the animals with progressive tumor growth. Furthermore, differences in the agglutinability of the red cells were found; animals showing low agglutinability were found to be most likely to produce isoagglutinins and likewise to be resistant to transplantation of the tumor.

Recently Gorer (11) reported on isoagglutinins which he produced in C57 Black and in C57 Brown either by injection of blood of strain A animals or by transplantation of two different tumors. He was able to demonstrate different types of antibodies, namely, agglutinins acting in saline, agglutinins acting only in serum medium, as well as blocking antibodies. These antibodies were produced in response to antigens present in the transplanted tumors and in the injected blood but absent in the immunized animals.

Reviewing the over-all results of the present study, it is felt that they present strong presumptive evidence for the assumption that strains of mice with high susceptibility to spontaneous development of tumors possess an impaired ability to produce natural and immune hemagglutinins and immune hemolysins. While the role of the reticulo-endothelial system in antibody production is by no means fully clarified, it appears possible that this immunologic deficiency of high tumor strains might be based on an impaired reticulo-endothelial function.

SUMMARY

1. Natural antisheep agglutinins were determined in the serum of mice of six inbred strains. A striking difference distinguished the C57 Black animals from animals of the other strains: in the former, antisheep agglutinins were found in more than 90 per cent of the animals, and titers of 16 and higher in more than half, whereas the incidence and the titers of the antibodies in the other strains were markedly lower.

2. Natural agglutinins for human red cells were present only infrequently and in low titers in the mouse serum, without any relationship to the strain of the animals tested.

3. In tumor-bearing animals of the C57 Black and of the C3H strains the distribution of antisheep agglutinins was similar to that in tumor-free animals, except that the presence of induced or transplanted tumors tended to raise the incidence and titer of agglutinins.

4. Immune antibodies against sheep red cells and human red cells were produced in C57 Black, C3H, and dba animals. Agglutinins and hemolysins for sheep red cells and human red cells reached higher levels in the majority of C57 Black animals, as compared with the values found in C3H and dba mice.

5. The antibodies were not absorbed by Forssman antigen. The significance of this finding was discussed.

6. The greater frequency of natural antisheep agglutinins, their significantly higher titers, and the more vigorous response to injections of sheep and human red blood cells, seem to justify the conclusion that mice of strain C57 Black have a more active and more responsive agglutinin-producing reticulo-endothelial system than the other five strains.

It would be tempting to correlate the apparent higher efficiency of the reticulo-endothelial tissues of strain C57 Black with the low incidence of spon-

taneous mammary carcinoma. However, it is equally possible that the two phenomena are not related to each other as cause and effect.

We hope that further study, especially of other strains with low tumor incidence and with high tumor incidence but free of the milk factor, may throw light on this problem.

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The Influence of Estrogen on Cancer Incidence and Adrenal Changes in Ovariectomized Mice on Calorie Restriction*

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The occurrence of mammary carcinoma (1, 2, 3, 4) following ovariectomy in certain strains of mice and the delay of mammary carcinogenesis in such mice by calorie underfeeding seen in preliminary experiments (5) suggested the testing of the effects of exogenous estrogen administration, particularly in the calorie restricted animal, on cancer production. Studies were therefore made on full-fed and calorie restricted ovariectomized mice, both with and without exogenous estrogen, observing effects upon the reproductive apparatus, the adrenal cortex, and upon mammary cancer incidence.

METHODS

C3H strain mice were ovariectomized at weaning (21 to 23 days) and caged individually in a room held at 78° ± 4° F. and 45 ± 10 per cent relative humidity. Groups as indicated were placed on full feeding and on 33 per cent calorie restriction, the diets being those described previously (6). In these diets the calorie restricted mice receive the same average daily intake of protein, vitamins, and minerals as their controls, but smaller amounts of carbohydrate and fat. Vaginal smears were studied in intervals; body weights were recorded and the mice were examined for tumors weekly. In some instances the animals were sacrificed for morphologic study when a mammary tumor was noted and in other instances the tumor was removed surgically under anesthesia and the mouse studied further as will be noted under results. The adrenal glands, the uteri, and the mammary tumors were sectioned and studied microscopically. In the estrogen treated animals 0.5 γ of diethylstilbestrol per day per mouse was administered in the food.

RESULTS

Table 1 presents a summary of the major findings in the study. It will be seen that the diethylstilbestrol-treated C3H castrates on full feeding show a markedly lower average cancer age than their controls without estrogen. This occurs with a total suppression of adrenal adenomatous change as would be expected from the report of Woolley and Little (7). The mice showed enlarged uteri and the vaginal wall and smears showed a high degree of estrogenic stimulation.

TABLE 1

THE INCIDENCE OF MAMMARY CARCINOMA ADRENAL ADENOMA IN OVARIECTOMIZED C3H MICE WITH AND WITHOUT EXOGENOUS ESTROGEN ON FULL FEEDING AND 33 PER CENT CALORIE RESTRICTION

No. of Mice	Feeding	Diethylstilbestrol γ per Mouse per Day	Body Wt. at 35 Wks. in Gms.	Per Cent with Mammary Tumors		Average Cancer Age (Months)	Per Cent with Adrenal Adenomata
				at 35 Wks.	at Death		
18	Full	0.0	29	28	94	11.1	100
15	Full	0.5	26	89	100	7.6	0
30	Restricted	0.0	18	0	0		100
8	Restricted	0.5	16	13	100	13.4	0

The calorie restricted ovariectomized C3H mouse showed a zero incidence of mammary cancer, while the adrenal adenomatous change was invariable in its occurrence. On the same diet with diethylstilbestrol the mammary cancer incidence became 100 per cent. However, the average cancer age was nearly double that in the fully fed estrogenized mice. The adrenal cortices in the calorie

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restricted estrogenized mice did not show adenomata. The accessory sex apparatus was similar to that in the full-fed mice.

In six cases the mammary tumors were surgically removed from calorie restricted estrogenized C3H mice and the administration of diethylstilbestrol stopped after 6 to 10 months of administration. The calorie restriction was continued. The animals were sacrificed after 1 month or more without estrogen. In each case adenoma of the adrenal cortex was found. Therefore it can be concluded that even after 10 months of diethylstilbestrol treatment the suppression of adrenal cortex adenoma by exogenous estrogen is capable of prompt reversal.

DISCUSSION

The observation that mammary cancer incidence in the calorie restricted ovariectomized C3H mice given exogenous estrogen may reach 100 per cent, as compared with zero without estrogen provides additional evidence that the pertinent end-organs are by no means insensitive to estrogen in the calorie restricted state. The sensitivity may not be as great as in the full-fed mouse, since there is a large difference in average cancer age.

It is significant in these results that calorie underfed ovariectomized C3H mice develop adrenal cortical adenomata indistinguishable histologically from those in fully fed animals. Nevertheless in the calorie underfed state the adrenal adenoma does not produce estrogen, which it is assumed to do in the fully fed castrate, as evidenced by the maintenance of a continuous subestrus state and by mammary gland and carcinoma development. The calorie underfed castrate does not show any of the latter changes except when exogenous estrogen is administered. It has been pointed out previously (8, 9) that calorie restriction appears to reduce the sensitivity of the mammary gland to estrogen. The lengthened average cancer age in these experiments may be due to this effect. There is no evidence as to how this effect is mediated but it might be through suppression of a pituitary hormone other than gonadotropin in calorie underfeeding.

The most significant point in these studies seems to be the confirmation they give to the hypothesis that calorie restriction in mice lowers mammary cancer incidence by reducing estrogen production 6, 8). There seems to be no other acceptable interpretation of the results as they appear. Any other interpretation would involve complicated postulates as to direct or remote (by way of the pituitary) effects of exogenous estrogen upon the adrenal cortex and these effects would have to be different in the full-fed and the calorie restricted animals. It therefore seems reasonably well established that the primary reason for suppression of mammary cancer in mice by calorie underfeeding is the suppression of estrogenic hormone production which it causes.

CONCLUSIONS

1. Ovariectomized C3H mice given 0.5 γ of diethylstilbestrol per day show an eventual 100 per cent incidence of mammary carcinoma regardless of whether the animals are fully fed or are restricted in calories to the extent that in the absence of exogenous estrogen the cancer incidence would have been zero.

2. The average cancer age in estrogen treated ovariectomized C3H mice is greatly increased by calorie restriction.

3. The observation that calorie restriction does not prevent estrogen induced mammary carcinoma in cancer susceptible mice lends added support to the hypothesis that calorie restriction reduces mammary cancer incidence in the intact or ovariectomized non-estrogenized mouse mainly by suppression of endogenous estrogen production.

4. The suppression of adrenal adenoma formation in ovariectomized C3H mice produced by exogenous estrogen is quickly reversible after 6 to 10 months of administration.

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The Products of Pyrolysis of Cholesterol at 360° C. and Their Relation to Carcinogens*

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Cholesterol has been used in many experiments as a vehicle in testing carcinogenicity of other compounds. It was adopted after tests on a small scale showed that it did not possess carcinogenic properties of its own. Hieger (13), however, on testing it in large numbers of animals detected definite, though slight, carcinogenic activity in some samples of commercial cholesterol. It has been suggested by many that cholesterol may be a factor in the causation of some human cancer, and Hieger (13A) further states that the carcinogenicity of the non-saponifiable lipid fractions of human livers seems to be concentrated in the portion containing a high proportion (85 per cent) of cholesterol.

It has been shown by Kennaway and Sampson (14) that cholesterol heated to 810° C. becomes carcinogenic. This temperature, however, is far above the range ordinarily reached in cooking. When cholesterol, or its esters, were heated to 200° C. and 300° C., a temperature sometimes used in cooking, few tumors were produced after

TABLE 1

PRODUCTS OF PYROLYSIS OF CHOLESTEROL AT 200° C. AND THEIR CARCINOGENICITY

Compound	Reference	Animals tested	Method of testing	No. of tumors
4-cholestenone	Kirby (15, 16)	Mice	Feeding	0
Dicholesteryl ether	Kirby (15, 16)	Mice	Feeding	0
5-cholestene	Barry (1)	20 mice	Skin painting	0
3,5-cholestadiene	Strong (27), Kirby (16)	Mice	Skin painting	0
			Subcutaneous	0
2,4-cholestadiene	Butenandt (4)	20 mice	Skin painting	0
2,4-cholestadiene peroxide	Butenandt (4)	20 mice	Skin painting	0

This carcinogenic activity associated with cholesterol might be due to four different causes: (a) the carcinogenic compound may be so similar to cholesterol in its chemical and physical properties that it might contaminate the commercial product; (b) it may be cholesterol itself, which, under pathological conditions in the organism, gives rise to a potent carcinogen like methylcholanthrene (8); (c) cholesterol itself might, under certain conditions, act as a carcinogen or as a cocarcinogen; or (d) the carcinogen may be a product of cholesterol formed prior to contact with the organism (3, 29). Of these, the latter possibility seems most probable.

The most likely agent causing this change of cholesterol to carcinogen would be heat. That cholesterol undergoes thermal decomposition is well known. The ingestion of these products in cooked foods offers a possible explanation for the high incidence of gastric and intestinal cancers in Europeans and Americans, as compared with the low incidence in primitive races of man and in all animal species that have been investigated.

skin painting or injection (2, 15 to 18, 26). At a temperature of 360° C., or above, cholesterol completely decomposes. The only experiments in this range were done by Kirby (17) who heated cholesterol to 430° C. and obtained three papillomas after skin painting with the products. From the ultraviolet absorption spectrum of the oil obtained, he deduced the presence of methylcyclopentenophenanthrene. This is presumably the phenanthrene derivative of cholesterol which Roffo (22, 23) claimed was carcinogenic. Not enough of this material was obtained by Kirby to test its carcinogenicity, and he relied on Cracium's claim of the carcinogenic activity of this compound (6).

Table 1 gives a compiled list of the compounds formed on the pyrolysis of cholesterol at a temperature of 200° C. or less. They are easily identifiable and they have been tested for carcinogenic-

* This investigation was assisted by a research grant from the American Cancer Society recommended by the Committee on Growth of the National Research Council.

ity. Of them only 3,5-cholestadiene has been claimed to have activity (28) but subsequent investigation found it to be inactive (16). The finding of Steiner *et al.* (26), of the lack of carcinogenicity of cholesterol heated to 200° C., confirms the results tabulated in Table 1. At higher temperatures pyrolysis of cholesterol gives rise to still other products. Above a certain critical temperature all the products listed in Table 1, with the exception of cholestenone, are no longer found as they have been decomposed.

The products of pyrolysis of cholesterol at temperatures above 300° C. but below the very high temperatures used by Kennaway and Sampson (14) and by Kirby (17) have not been thoroughly studied. It is in this range that tremendous changes take place in the cholesterol molecule—a range that can occur under conditions sometimes encountered in cooking. It is important to know whether these changes include the formation of carcinogenic compounds. Therefore, an investigation involving the identification and biological testing for carcinogenic activity of the products of cholesterol heated to 360° C. was undertaken. The results of the former are here reported.

EXPERIMENTAL PROCEDURES

A. Apparatus for pyrolysis.—A 300 ml. standard taper, flat-bottom flask was connected by means of two adapters to a standard taper, round-bottom flask. The first adapter held a thermometer which reached to the bottom of the flask and the second adapter had an air outlet. The flat-bottom flask of this all glass apparatus was heated in a sandbath by an electric hot-plate.

B. Heating procedure.—The electric hot-plate was adjusted to full heat. As soon as the sandbath reached 250° C., the flask containing 5 grams of commercial cholesterol was placed in the sand. In about 5 minutes the temperature inside the flask reached 340° C., and the hot-plate was then regulated to maintain a temperature of 360° C. to 380° C. for 30 minutes. The apparatus was then removed and allowed to cool.

C. Chromatography.—All the heated cholesterol products were chromatographed on a column of activated aluminum oxide (Alcoa, especially prepared for chromatography) prepared in the following way. A dried glass tube, 2 cm. × 60 cm., was tapered at one end and stoppered with glass wool. About 120 gm. of alumina was poured into it and the column was washed with redistilled, dried petrol ether (b.p. 30°–60° C.). The cholesterol products were dissolved in petrol ether, poured on the column, and eluted with increasing concentrations of pure anhydrous ether in petrol ether. All the fractions were taken to dryness on the hot water bath, tested with ultraviolet light for fluorescence, transferred to a separate flask and reevaporated under nitrogen.

D. Spectrophotometry.—With ether as solvent, each fraction was analyzed for its ultraviolet absorption spectrum in a Beckman Quartz Spectrophotometer, between the wave lengths of 215 and 400 mμ. Readings were taken every two to five mμ as indicated by the individual spectrum.

EXPERIMENTS AND RESULTS

Experiment 1. Absorption spectra of commercial cholesterol.—Cholesterol[1] was chromatographed and analyzed in the spectrophotometer in search of identifiable compounds before heat was applied. No aromatic hydrocarbons were found, but small amounts of ergosterol were detected. Cholesterol obtained from Wilson and Co., Chicago, Ill., gave similar results but it contained larger quantities of ergosterol.

Experiment 2. Chromatography of methylcholanthrene and the limits of its detection.—The purpose of this experiment was to determine the quantitative recovery of methylcholanthrene, its elution pattern, its precise ultraviolet absorption spectrum, and the minimal quantity detectable by this method. Methylcholanthrene (2.9 mg.) was dissolved in petrol ether and chromatographed on aluminum oxide. Eighty-three per cent was recovered in the eluates containing 25 per cent ether in petrol ether. The limit of detection of methylcholanthrene was found to be 0.1 μg. In the presence of impurities encountered in these fractions 5 μg. of methylcholanthrene could be detected in a solution containing 1000 μg. of impurities. The presence of 100 μg. of methylcholanthrene in 5 gm. of the pyrolysis products can therefore be detected by this method.

Experiment 3. Choice of atmospheric conditions during pyrolysis.—Five gram samples of cholesterol were heated at 360° C. for 30 minutes (a) in an open vessel, (b) under a stream of nitrogen, (c) *in vacuo*, and (d) in the standard apparatus as described above. In the open vessel with unlimited oxygen supply the product of heating was a black tar which was only slightly soluble in petrol ether, partly soluble in ether, and completely soluble in chloroform. Since chromatography of this product was not practical and since carcinogenic hydrocarbons are all soluble in petrol ether, this procedure was not further used. Cholesterol, heated under a stream of nitrogen, failed to reach the desired temperature. When heating was carried out *in vacuo* much of the starting material was lost as the dicholesteryl ether. Thus the procedure with limited oxygen supply was adopted as standard.

Experiment 4. Thirty minute pyrolysis of cholesterol at 360° C.—Ten grams of cholesterol were heated at 360° C. for 30 minutes. A dark amber oil

[1] Unless otherwise stated the cholesterol used was from a single batch obtained from the Armour Laboratories, Chicago.

was produced which gave a strong blue fluorescence under ultraviolet light. This oil was chromatographed; the results are given in Table 2. Each fraction was then analyzed in the spectrophotometer for ultraviolet absorption spectra. Fractions 1 and 2 gave a nondescript spectrum which is not illustrated here. It was a colorless nonfluorescent oil, a mixture of cholestane and coprostane. Fractions 3 to 7 were colorless oils with slight blue fluorescence; they gave a spectrum as shown in Figure 4, identified as the naphthalene derivative of cholesterol. Fractions 10 to 16 were pale yellow oils giving strong blue fluorescence and yielding crystals with a spectrum as shown in Figure 9. In later, more polar fractions, a white, crystalline, nonfluorescent compound was eluted. This was identified as cholesterol. The most polar fractions contained brown oily crystals whose absorption spectrum was similar to that found in experiment 4 and which is illustrated in Figure 8. All other characteristic curves found in experiment 4 were absent.

Experiment 6. Thirty minute pyrolysis of cholesterol at 400° C.—The products formed differed in no discernible way from those produced in experiment 4 at 360° C.

TABLE 2

RESULTS OF CHROMATOGRAMS IN TWO EXPERIMENTS

		Experiment 3. 30 Minute Pyrolysis			Experiment 6. 4 Hour Pyrolysis		
			Eluate			*Eluate*	
No. of fraction	Eluant (100 ml.)	Wt. in mg.	Physical properties	Chemical structure	Wt. in mg.	Physical properties	Chemical structure
1	100% ligroin	2263	Colorless oil	IV	3700	Colorless oil	IV
2	"	1744	No fluorescence		1166	No fluorescence	
3	1% ether*	460			330		
4	"	289	Colorless oil		213	Colorless oil	
5	2% ether	391	Slight blue fluorescence	VI	97	Slight blue fluorescence	VI
6	"	138	Abs. spectrum		101	Abs. spectrum	
7	4% ether	94	Fig. 4			Fig. 4	
8	"	123			92		VII
9	6% ether	40			117		VII
10	"	79					
11	10% ether	63				Colorless oil	
12	"	97			83	Blue fluorescence	IX
13	"	32	Pale yellow oil		158	Abs. spectrum†	
14	15% ether	31	Blue fluorescence	VII		Fig. 6	VII
15	"	36	Abs. spectrum		89		VII
16	"	7	Fig. 6		91		VII
17	20% ether	20					
18	"	36	Pale yellow oil		113	Pale yellow oil	
19	30% ether	33	Blue fluorescence	V	67	Blue fluorescence	V
20	"	50	Abs. spectrum Fig. 7			Abs. spectrum	
21	50% ether	47				Fig. 7	
22	"	66			150		
23	100% ether	145					
24	"	375	Brown oil. No fluorescence	XIII			
25	100% methanol	774	Abs. spectrum Fig. 8				

* These figures in fractions 3 to 22 refer to percentage of ether in ligroin.
† Fraction 11 contained crystals.

the spectrum, shown in Figure 6, of the phenanthrene derivative of cholesterol. Fractions 17 and 18 gave a yellow oil with a spectrum as shown in Figure 7, probably cholesterol with ring A or B aromatic. Fractions 24 and 25 were brown oils with the spectrum shown in Figure 8, typical of α, β-unsaturated ketones like cholestenone. The fractions between those described gave either noncharacteristic curves or composites of two curves.

Experiment 5. Thirty minute pyrolysis of cholesterol at 340° C.—The cholesterol after having been heated at this lower temperature was a light amber oil which contained crystals. It showed slight fluorescence. Chromatographic analysis of this product revealed in the first eluate the presence of

Experiment 7. Variations in duration of pyrolysis.—Ten gram samples of cholesterol were heated at 360° C. for 1, 2, 4, and 8 hours. A dark amber oil was obtained by each heating. Chromatography revealed only quantitative differences from those of experiment 4 (Table 2). Most important of these quantitative differences was the increase in the colorless, nonfluorescent oily fractions 1 and 2, a decrease in the fractions giving the spectrum of Figure 4, and an increase in the quantity of material which had the spectrum of Figure 6. Also, in the later fractions there was a great increase in the amount of crystals giving the spectrum of Figure 6. Spectrophotometry revealed the same curves as those found in experiment 4 except for the 8 hour

pyrolysis which gave one new compound. This was found in the 20 to 50 per cent ether eluates; it is shown in Figure 11, the spectrum of chrysene.

Experiment 8. Pyrolysis of cholesterol with selenium.—Five grams of cholesterol combined with 782 mg. of selenium were heated in the usual apparatus for one hour at 360° C. under a hood. The resultant oil was not different, grossly or on analysis, from that obtained in the 4 hour pyrolysis in experiment 7.

Experiment 9. Thirty minute pyrolysis of cholesterol rich in ergosterol at 360° C.—The results with a cholesterol which is rich in ergosterol obtained from another source (Wilson and Co.) did not differ from those of experiment 4.

Experiment 10. Chemical separation.—The amber fluorescent oil obtained on pyrolysis of 5 grams of cholesterol as in experiment 4 was dissolved in ether (100 ml.) and extracted with 10 per cent sodium carbonate solution (3 × 30 ml.) to remove all acids. The ether solution was washed until it was neutral, dried with sodium sulfate, and evaporated to dryness. An oily residue was obtained. The original ether solution was then extracted with *N* sodium hydroxide solution (3 × 30 ml.) to remove all phenols and enols. The extract was treated like the acids described above. The remaining ether solution was washed until neutral, dried with sodium sulfate and evaporated to dryness. This neutral material was then separated into ketonic and non-ketonic fractions by means of Girard's reagent T (21). An attempt was made to remove aromatic hydrocarbons from the non-ketonic neutral fraction as the picrate. However, the only crystals isolated were those of picric acid.

Experiment 4 was repeated several times to ascertain the reproducibility of the results and to obtain larger quantities for bioassay. The results were always the same.

INTERPRETATION AND DISCUSSION

A. Identification of the compounds.—Identification of the products of pyrolysis by chemical means was not undertaken because the crystalline products did not hold enough interest for this investigation and the products of importance were oils which could not be purified readily, but whose absorption spectra were sufficiently specific to serve for their tentative identification. In interpreting ultraviolet absorption spectra it must be remembered that they are produced by the absorption of light by resonating electrons in a molecule and that substitutions only in key positions in the molecule will shift the absorption peaks towards longer wave lengths. Minor changes in the structure of the molecule at a distance from the resonating electrons can therefore not be detected by this means, unless this change produces new resonating structures.

For identification of the absorption spectra of the pyrolytic products of cholesterol reference was made to the work of Mayneord and Roe (20). Figure 3 shows their absorption spectrum of naphthalene and the shift produced in the spectrum by the addition of aliphatic groups to this resonating structure. Figure 4 shows the spectrum and tentative structure of a compound found in all pyrolysis experiments on cholesterol in the range of 360° C. here described. It has been identified as the naphthalene derivative of cholesterol.

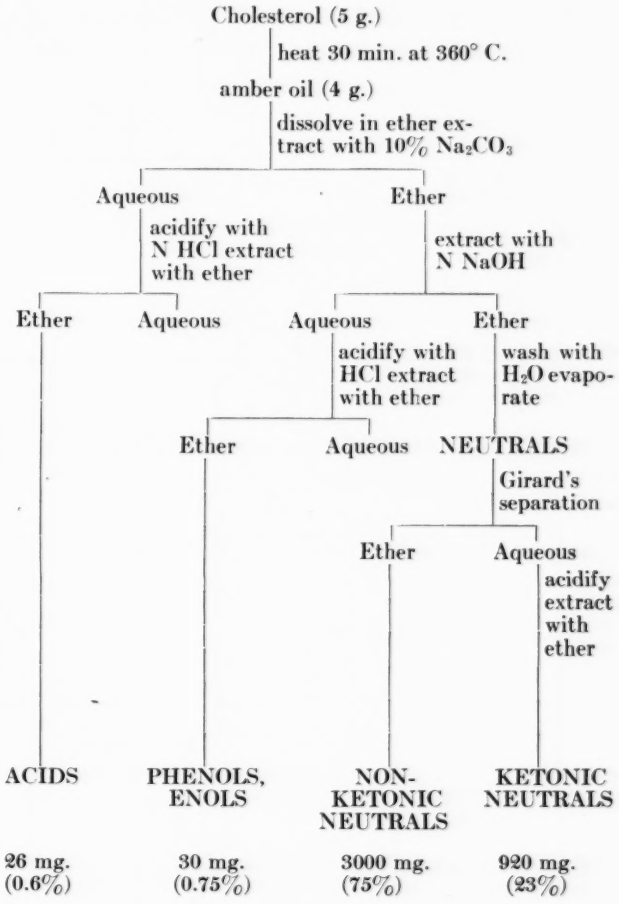

FIG. 1.—Chemical separation of the products of pyrolysis of cholesterol.

Figure 5 gives the spectrum of pure phenanthrene and of cyclopentenophenanthrene (20). The latter as well as the phenanthrene derivative of cholesterol were found in all pyrolysis experiments of cholesterol reaching 360° C. (Fig. 6).

The absorption curve given in Figure 7 was found in all experiments of pyrolysis at 360° C. It is too nondescript for identification. It could be given by compounds possessing one aromatic ring

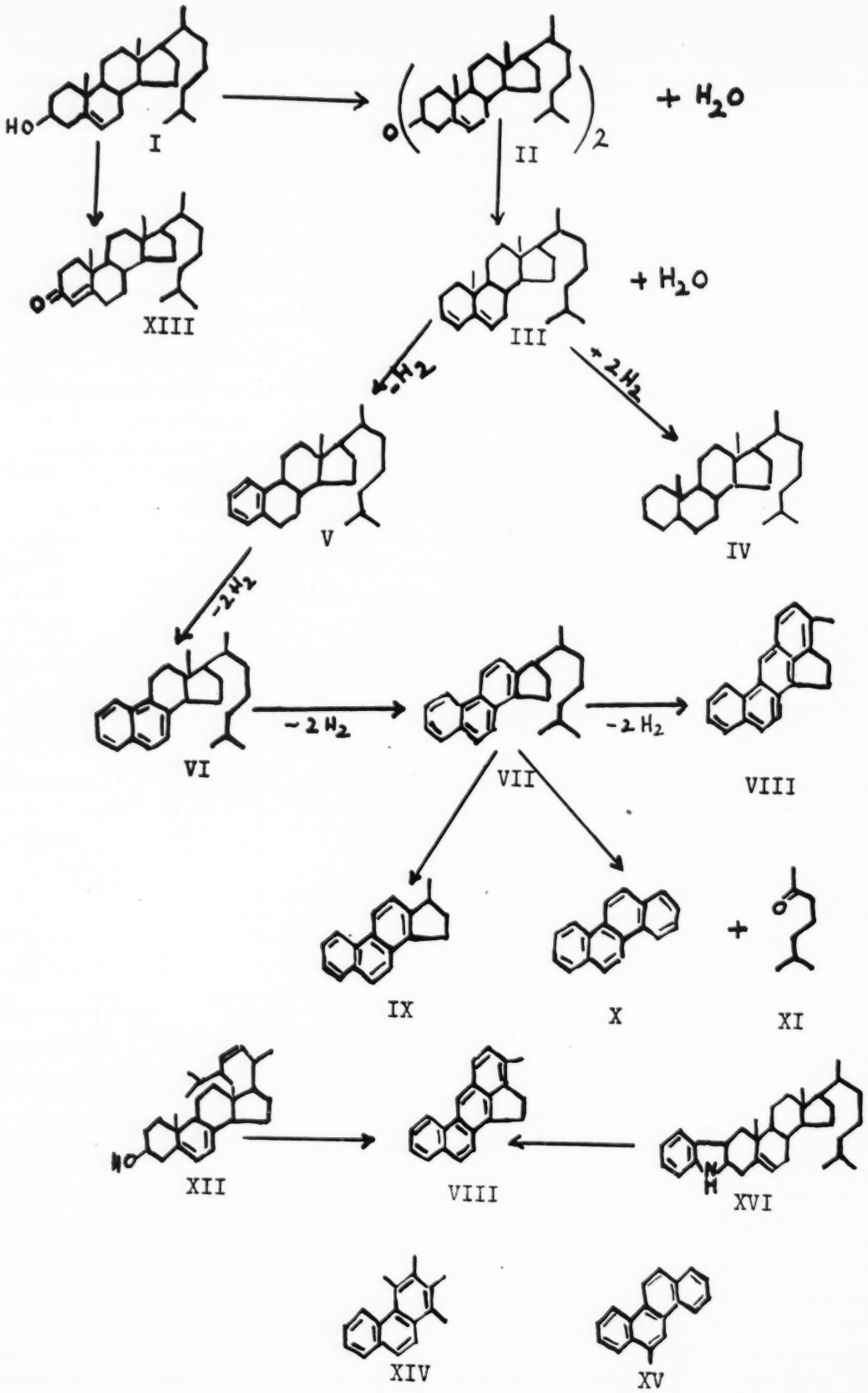

FIG. 2.—Products of pyrolysis of cholesterol; related compounds. Compound I, Cholesterol; II, Dicholesteryl ether; III, 3,5-cholestadiene; IV, Cholestane and coprostane; V, Benzene derivative of cholesterol; VI, Naphthalene derivative of cholesterol; VII, Phenanthrene derivative of cholesterol; VIII, Methylcholanthrene; IX, Methylcyclopentenophenanthrene; X, Chrysene; XI, Methyl-isohexyl ketone; XII, Ergosterol; XIII, Cholestenone; XIV, 1,2,3,4-tetramethylphenanthrene; XV, 6-methylchrysene; XVI, Indole derivative of cholesterol.

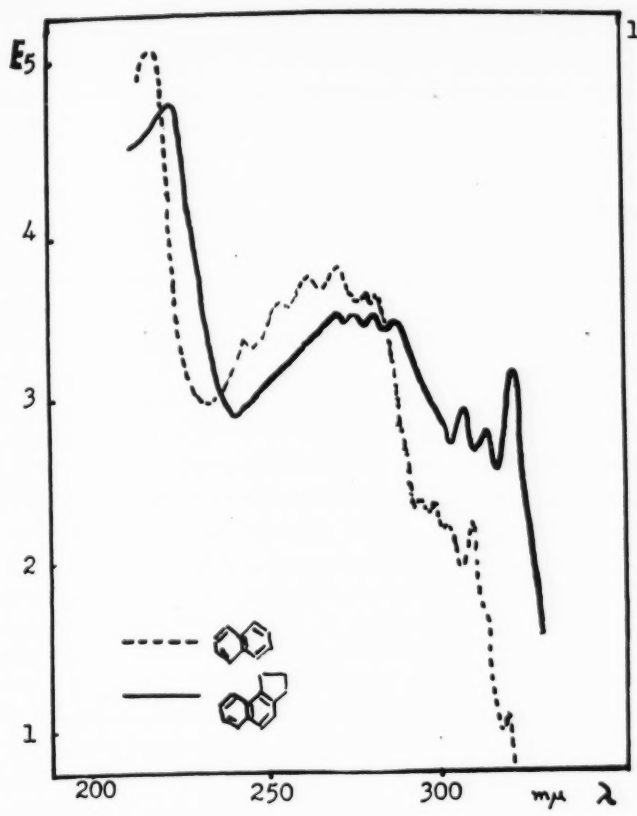

FIG. 3.—Absorption spectra of naphthalene and 1,2-cyclopentenonaphthalene

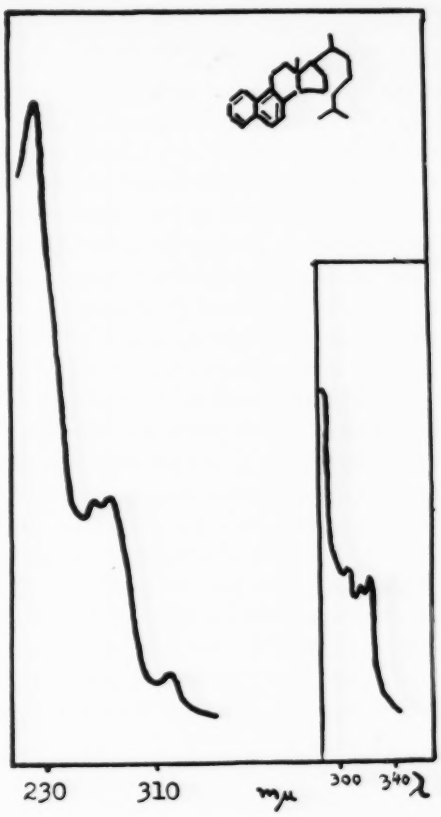

FIG. 4.—Absorption spectrum of the naphthalene derivative of cholesterol.

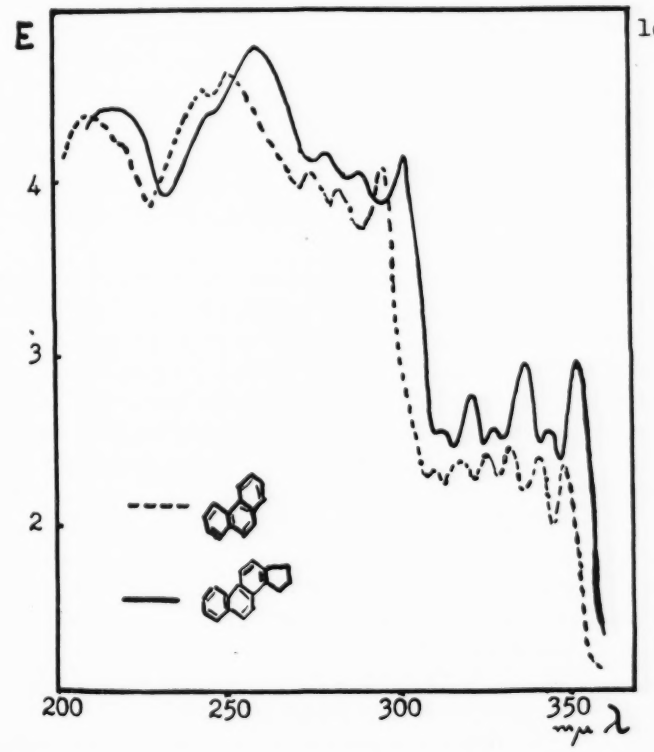

FIG. 5.—Absorption spectra of phenanthrene and 3,4-cyclopentenophenanthrene.

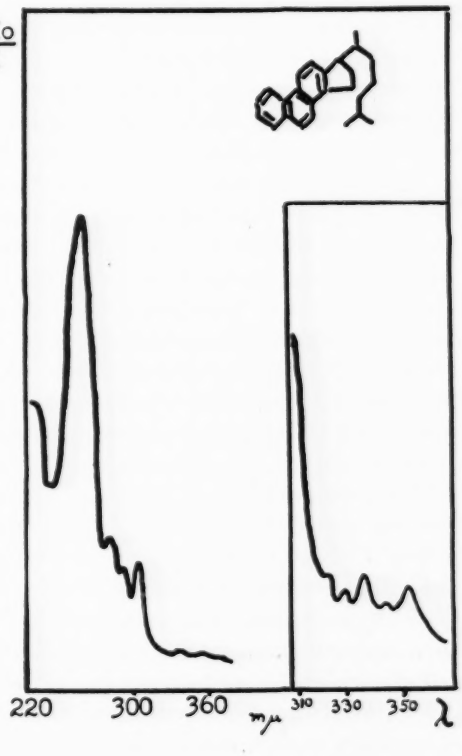

FIG. 6.—Absorption spectrum of the phenanthrene derivative of cholesterol.

as diagrammed. The lack of specificity of the spectra of compounds with one aromatic ring prevents their positive identification. The curve shown in Figure 8, identified tentatively as cholestenone, was eluted in the more polar fractions; it was present in all pyrolysates of cholesterol regardless of the temperature used. Another nondescript curve is given in Figure 9. Many compounds with conjugated double bonds show this absorption peak. However, a tentative identification of this compound as 3,5-cholestadiene can be made because of (a) its color reaction with antimony trichloride, (b) its presence in only the first eluates, (c) its presence only in experiments in which the temperature was kept at 340° C. (experiment 5), and (d) its absorption spectrum (7, 9, 25, 30). The absorption spectrum of pure chrysene and of the chrysene derivative found in experiment 7 (pyrolysis at 360° C. for 8 hours) is given in Figures 10 and 11. Figure 12 gives the curve of methylcholanthrene which was not found in any of the experiments.

Chemical separation was undertaken to estimate the proportion of various chemical groups among the products. It was found (Fig. 1) that the acids, phenols, and enols were negligible, and that 23 per cent of the neutral material was ketonic and that 75 per cent was non-ketonic.

B. Degradation of cholesterol and the possible transformation to methylcholanthrene.—Fieser states (10) that aromatization of the ring system and cyclization of the sidechain of cholesterol would lead to methylcholanthrene. All but the final step —cyclization of the sidechain—have been shown to occur in these experiments. The changes are diagrammed in Figure 2. The process starts with the removal of one molecule of water from 2 molecules of cholesterol (compound I) forming the dicholesteryl ether (compound II) (19). Crystals of dicholesteryl ether sublimed on the wall of the adapter and decomposed when the temperature rose above 200° C. Another molecule of water was removed, forming 3,5-cholestadiene (compound III). This compound was found as a crystalline product on pyrolysis at 340° C., but not at or above 360° C. The next step was a coupled reduction and dehydrogenation in which the hydrogen from 3,5-cholestadiene was removed to form the benzene, naphthalene, and phenanthrene derivatives of cholesterol (compounds V, VI, and VII). At the same time the hydrogen liberated was added to other molecules of 3,5-cholestadiene giving rise to saturated hydrocarbons such as cholestane and coprostane (compound IV). As the time of heating was increased, more of this colorless, saturated hydrocarbon was formed. Compound V, the benzene derivative, cannot be definitely identified by its absorption spectrum, as was stated above. Compound VI, the naphthalene derivative, and compound VII, the phenanthrene derivative of cholesterol, however, possess characteristic spectra. Instead of cyclization of the sidechain, the next step consisted of a breaking off of the sidechain to form the crystalline methylcyclopentenophenanthrene (compound IX) in all pyrolyses at 360° C. A chrysene derivative (compound X) was obtained when pyrolysis was continued for 8 hours. This change of a five membered ring to a six membered ring is a well known reaction of steroid molecules. The step leading to the formation of methylcholanthrene was not found.

For this reason the selenium dehydrogenation and the pyrolysis of cholesterol which contained much ergosterol (experiment 9) were undertaken. Rossner (24) described the formation of methylcholanthrene from an indole derivative of cholesterol (compound XVI) by pyrolysis for a total of 70 hours at 320° and 340° C. followed by 9 hours heating at 360° C. with selenium as a catalyst. Cholesterol was heated with selenium (experiment 8) to see whether selenium was the only missing requirement to obtain cyclization of the sidechain. However, not methylcholanthrene but only larger quantities of methylcyclopentenophenanthrene were obtained by this procedure. Cholesterol having a high content of ergosterol (compound XII) was used in experiment 9 for pyrolysis. It was assumed that cyclization would be easier due to the double bond in the $C_{22, 23}$ position of the sidechain. Again, however, methylcholanthrene was not obtained.

The duration of heating was found to be an important factor in determining the products formed. Chrysene was found only on 8 hour pyrolysis and larger quantities of cyclopentenophenanthrene were found as pyrolysis was prolonged. The temperature was also of importance because none of the structures with 2, 3, or 4 aromatic rings were found when the heating was carried out below 360° C. The presence of selenium gave the same products as were obtained by ordinary pyrolysis over a longer period of time; it increased the speed of reaction. Another product of pyrolysis which was detected at all times was 4-cholestenone (compound XIII) (12, 31). It played no part in the chain of reactions under investigation.

C. Other compounds with possible carcinogenic activity.—The absence of evidence for the formation of methylcholanthrene or of other known carcinogens does not rule out the possibility of

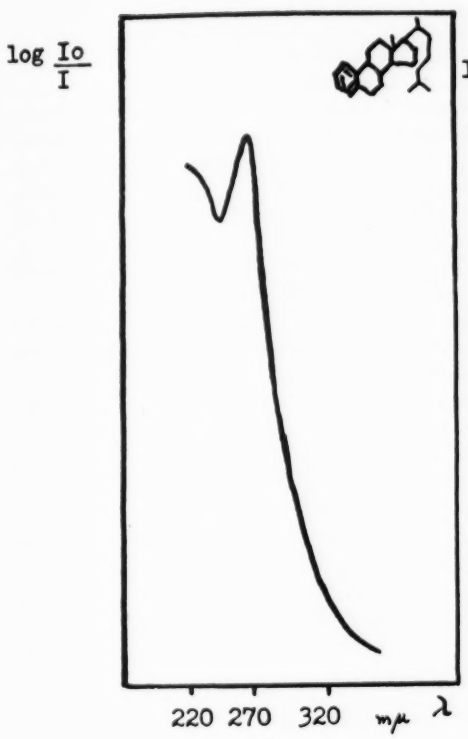

FIG. 7

FIG. 7.—Absorption spectrum of the benzene derivative of cholesterol.

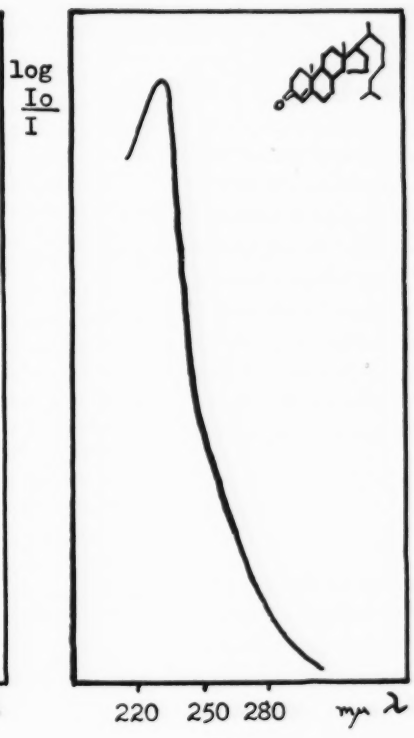

FIG. 8

FIG. 8.—Absorption spectrum of cholestenone

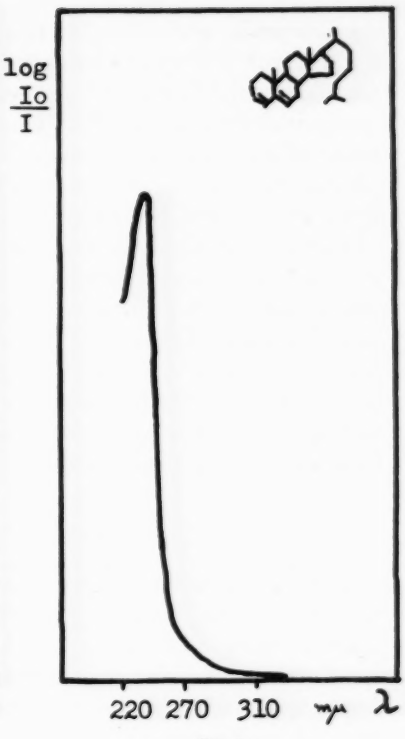

FIG. 9

FIG. 9.—Absorption spectrum of 3,5-cholestadiene

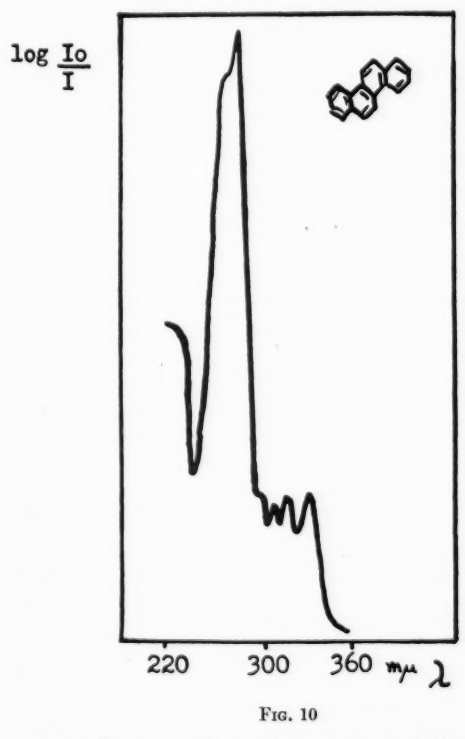

FIG. 10

FIG. 10.—Absorption spectrum of chrysene

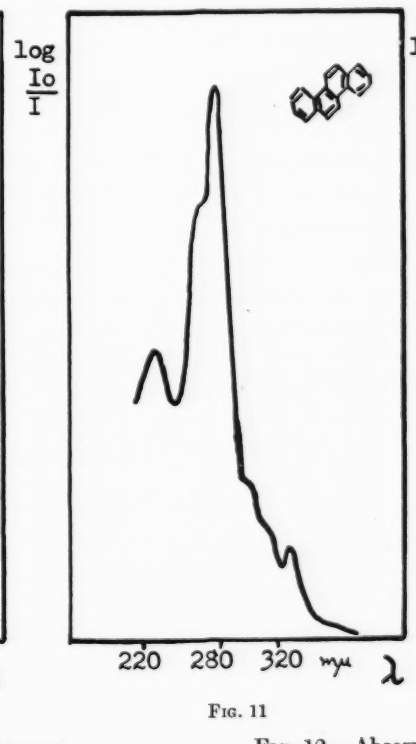

FIG. 11

FIG. 11.—Absorption spectrum of impure chrysene obtained by pyrolysis of cholesterol.

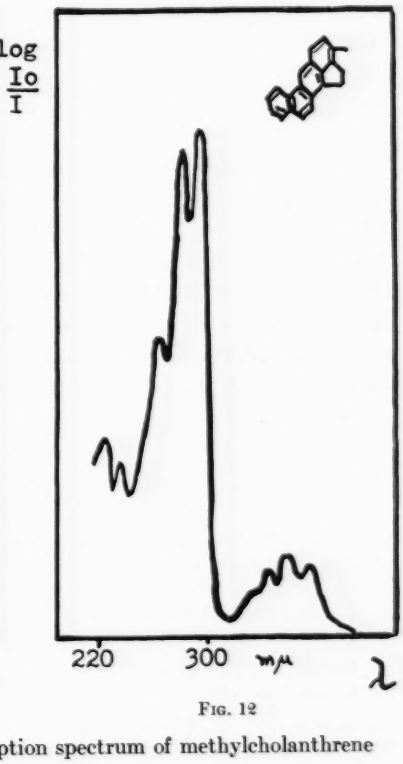

FIG. 12

FIG. 12.—Absorption spectrum of methylcholanthrene

carcinogenic activity by pyrolysis products of cholesterol. Methylcyclopentenophenanthrene was found in abundance and the reported evidence for or against its carcinogenicity is inconclusive. A related three ring compound 1,2,3,4-tetramethylphenanthrene (compound XIV) has been shown to have weak carcinogenic activity (5) and a number of chrysene derivatives possess similar weak activity (11). The possibility that methylcholanthrene was produced in quantities too small to be detected is still present because all steps except one in its formation from cholesterol were detected. Final conclusions as to the carcinogenicity of the individual pyrolysis products of cholesterol must await bioassay, which is under way.

SUMMARY

The products formed by pyrolysis of cholesterol at 360° C. were separated and the following compounds were identified: cholestenone, dicholesteryl ether, 3,5-cholestadiene, the naphthalene derivative of cholesterol, the phenanthrene derivative of cholesterol, methylcyclopentenophenanthrene, and chrysene.

Except for cholestenone all of these products are intermediates in the transformation of cholesterol to methylcholanthrene. The latter compound was not found because instead of cyclization of the sidechain a break occurred at the C_{17} position. The dehydration and dehydrogenation of the ring system occurred but the last step, cyclization of the sidechain, did not.

Six compounds whose degree of carcinogenic activity is unknown or uncertain were obtained. These include the saturated hydrocarbons cholestane and coprostane, the naphthalene derivative of cholesterol, the phenanthrene derivative of cholesterol, methylcyclopentenophenanthrene, the chrysene derivative, and the ketonic products. Tests for carcinogenicity of these products are under way.

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New Books

Neoplasms of Bone and Related Conditions. By BRADLEY L. COLEY, M.D.; Attending Surgeon, Bone Tumor Department, Memorial Hospital for Cancer and Allied Diseases, Assistant Professor of Clinical Surgery, Cornell. New York: Paul B. Hoeber, Inc., 1949. Pp. 779 + 622 illus. $17.50.

This new textbook is compiled largely from the author's large experience with this group of diseases. It is written for all clinicians who see bone tumors and related diseases. Numerous excellent x-rays, photographs, and photomicrographs illustrate almost every type of these diseases. The text is divided into eleven sections each with its own extensive, although not exhaustive, bibliography. The descriptions are excellent but sometimes too brief, since there is page space to spare. All clinical aspects are covered including the difficult problems of differential diagnosis and treatment. This is an excellent practical book which can be recommended to clinicians.

Techniques of Histo- and Cytochemistry: A Manual of Morphological and Quantitative Micromethods for Inorganic, Organic, and Enzyme Constituents in Biological Materials. By DAVID GLICK, PH.D.; Associate Professor of Physiological Chemistry, The Medical School, University of Minnesota. New York–London: Interscience Publishers, Inc., 1949. Pp. 531 + 159 illus. $8.00.

The subtitle aptly describes the contents of this welcome reference book. In it many of the most valuable technics have been collected. They are grouped into four sections namely microscopic, chemical, and microbiological techniques, and mechanical separation of cellular components. Some readers might desire more information about the relative merits of different methods, and others might want more discussion of interpretation of results. The author has admirably succeeded in his objective of presenting the technics and devices by which chemical investigation can be brought directly to the cell. The book can be highly recommended.

Announcements

INTERNATIONAL CANCER RESEARCH COMMISSION will meet in Paris July, 18 to 22, 1949. This organization was established at the Fourth International Cancer Research Congress in St. Louis in 1947. Dr. Ignacio Millan, Avenida Veracruz 69, Mexico, D.F., is the Chairman. At present 45 countries are represented on the Commission. The representatives change at regular intervals and the country in which the meeting takes place changes every year.

FIFTH INTERNATIONAL CANCER RESEARCH CONGRESS, under the Presidency of Dr. A. Lacassagne, will be held in Paris, July 17 to 22, 1950. During the mornings symposia will be presented on the Biology of Cancer. In the afternoons the papers presented will be grouped in the following sections:

1. Experimental investigations on cancer
2. Cancer of nervous system
3. Cancer of gastro-intestinal tract
4. Cancer of urogenital apparatus
5. Cancer of the respiratory apparatus
6. Cancer of tegument, breast, and endocrines
7. Cancer of skeleton and limbs, of blood and reticulo-endothelial system
8. Social fight against cancer

Details can be obtained from Dr. V. Le Lorier, Secretary, Union Internationale Contre le Cancer, 6 Avenue Marceau, Paris, France.

NOTE ON PUBLICATION: Transactions of the Fourth International Cancer Congress are in process of publication in 5 parts. The first part appeared in 1948 as No. 1, Vol. 6 of ACTA (Union Internationale Contre le Cancer) under the editorship of Dr. J. H. Maisin. It amounts to 267 pages including illustrations, tables, and brief summaries of all papers in English, French, German, Italian, Russian, and Spanish. The second part has probably also come out. It is expected that the remaining ones will appear in rapid succession. The price for the complete Transactions, set by Dr. J. H. Maisin, is $25.00, that is $5.00 for each of the 5 parts. Subscriptions should be sent as Postal Money Orders to: Dr. J. H. Maisin, 61 Voer des Capucins, Louvain, Belgium. Since an edition of only 500 copies of the Transactions is being printed, individuals wanting copies should subscribe promptly.

Reprints of the papers published in the Transactions can also be obtained from Dr. Maisin. It is suggested that those desiring reprints communicate directly with him. He will know the number of pages of the particular paper and will give the cost of the number wanted.